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CLINICAL TRANSLATION OF A REGENERATION STRATEGY FOR SPINAL CORD INJURY

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CLINICAL TRANSLATION OF A REGENERATION STRATEGY FOR SPINAL CORD INJURY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my Family

ABSTRACT

The complex and vulnerable tissue of the spinal cord does not heal after injury, leaving patients with lifelong disability after spinal cord injury (SCI). Many milestones have been reached during the last century through specialized centers for SCI, greatly increasing life expectancy and quality of life by battling common medical problems such as urinary tract infections, pressure ulcers, spasticity, neurogenic pain, and sexual function as well as providing means of rehabilitation to a meaningful and productive life after SCI. Despite the advances in preclinical knowledge of mechanisms in SCI and several clinical trials completed, to date no pivotal treatment exists for acute spinal cord injury or for the regeneration of lost function in the chronic state. The first reports of experimental regeneration of central axons through peripheral nerve grafts are more than a century old. In the last decades, regeneration of function after SCI has been reported by several research groups in different species using peripheral nerve grafts and FGF1. The regeneration strategy was furthered refined in our group by the use of a biodegradable scaffold for exact positioning of the nerve grafts. This thesis describes the translational process to reach a clinical trial of glial scar resection and implantation of peripheral nerve grafts and FGF1 using a biodegradable guiding scaffold.

In paper I, we show that both the cranial and caudal demarcation of a thoracic spinal cord injury can be defined with electromyography of intercostal muscles in chronic SCI patients. We also present an MRI protocol with acceptable image contrast despite the presence of spinal instrumentation and showed that the injury length found with electromyography correlates well with length of injury on MRI.

In paper II, we use a novel conversion table between spinal cord neuronal segments and vertebral segments and combine data on human spinal cord cross-sectional diameters from different published sources to yield continuous estimates on human spinal cord size and variability.

In paper III, we describe the design of a set of spinal cord injury guiding devices based on the data from paper II, covering the normal variability found in human thoracic spinal cord segments T2–T12 with an acceptable error-of-fit for the elliptical shape as well as guiding channels proposed.

In paper IV, we detail the adverse events reported during the first 60 days postoperatively in the ongoing clinical trial "Safety and Efficacy of SC0806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects." Early results from the first six complete (AIS-A) thoracic spinal cord injury subjects operated on in the ongoing trial show that with precise preoperative and intraoperative neurophysiology, surgery and implantation can be performed without negative effects on neurological level, and safety and tolerability are acceptable to merit the continuation of the trial.

In paper V, we describe the construction of a cost-effective light-sheet microscope by modification of an outdated microarray-scanner. The microscope was applied to an experimental model of hypoglossal nerve avulsion injury, and proliferation of Iba1+ cells could be quantified automatically demonstrating a possible application of the microscope.

In conclusion, reaching clinical trial in a translational process is a significant and collaborative undertaking requiring co-operation of multiple institutions and professions as well as rigorous external control of data quality and adverse events to ensure safety of study subjects. The papers in this thesis detail some relevant steps necessary for the clinical translation of regeneration strategies in chronic SCI.

LIST OF SCIENTIFIC PAPERS

I. Neurophysiological evaluation of segmental motor neuron function of the thoracic cord in chronic SCI.
Arvid Frostell, Per Mattsson, Jonas K.E. Persson, Björn Hedman, Jonathan Nordblom, Anders Lindenryd, Katarzyna Trok, Lou Brundin, Mikael Svensson
Spinal Cord. 2012 Apr 20;50(4):315–9.

II. A Review of the Segmental Diameter of the Healthy Human Spinal Cord.
Arvid Frostell, Ramil Hakim, Eric P. Thelin, Per Mattsson, Mikael Svensson
Front Neurol. 2016 Dec 23;7:238.

III. Guiding Device for Precision Grafting of Peripheral Nerves in Complete Thoracic Spinal Cord Injury: Design and Sizing for Clinical Trial.
Arvid Frostell, Per Mattsson, Mikael Svensson
Front Neurol. 2018 May 22;9:356.

IV. A Report of Adverse Events from an Ongoing Clinical Trial Evaluating Glial Scar Resection and Implantation of Nerve Grafts and FGF1 using a Biodegradable Scaffold in Chronic Traumatic Spinal Cord Injury
Per Mattsson, Arvid Frostell, Ann-Christin von Vogelsang, Jonas K.E. Persson, Hans Basun, Björn Hedman, Lou Brundin, Mikael Svensson
Manuscript

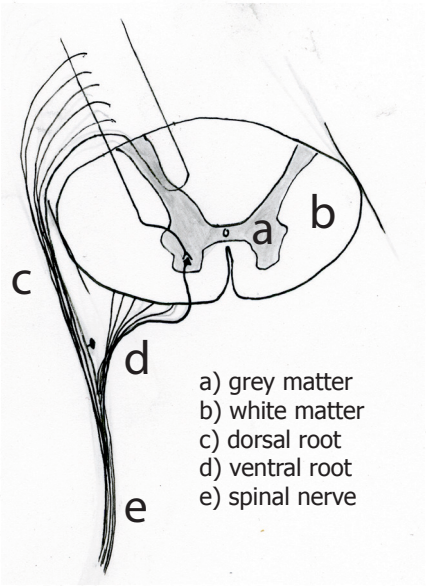
V. Light-Sheet Microscopy Using an Outdated Microarray Scanner and a Consumer Digital Camera
Arvid Frostell, Pendar Khalili, Ramil Hakim, Per Mattsson, Lou Brundin, Mikael Svensson
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AIS	ASIA Impairment Scale
ASIA	American Spinal Injury Association
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computed Tomography
DMC	Data Monitoring Committee
EMG	Electromyography
FGF1	Fibroblast Growth Factor 1
fMRI	Functional MRI
HRQoL	Health Related Quality of Life
ISCOS	International Spinal Cord Society
ISNCSCI	International Standards for Classification of Spinal Cord Injury
LSFM	Light-Sheet Fluorescence Microscopy
MEP	Motor-Evoked Potential
MRI	Magnetic Resonance Imaging
NLI	Neurological Level of Injury
SCI	Spinal Cord Injury
SEP	Sensory-Evoked Potential

THE SPINAL CORD



Cervical
C1-C8

Respiration
C3-C5

Arm/Hand
C5-T1

Thoracic
T1-T12

Trunk
T1-L1

Lumbar
L1-L5

Sacral
S1-S5

Leg
L2-S2

Urogenital
S2-S5

THE SPINAL CORD AND MICROSCOPIC INVESTIGATION

THE SPINAL CORD IS AN EXTENSION OF THE BRAIN

The spinal cord in humans is a thin structure that extends from the base of the skull via the neck to halfway through the torso. The spinal cord resides inside the spinal canal of the spinal vertebrae. The spinal cord contains most of the connections between the brain and the body, such as the conscious instruction to perform a voluntary movement of a finger to touch an object and the sensory information from the fingertip telling the brain of the tactile properties of the object (1).

However, the spinal cord is *not* just a cable between the brain and the body. It also contains many important modulations of the information passed to and from the brain. Motor neurons in the spinal cord take input from the brain, amplify the electrical signal, and directly activate muscles. The sensory neurons of the dorsal ganglia (situated *outside* spinal cord) modulate and pass information to the brainstem via the spinal cord but also connect directly to other neurons of the spinal cord.

The spinal cord can even perform tasks entirely on its own such as the extensor reflex (the jerk of the leg that medical doctors examine with a reflex hammer). This activation of motor neurons and subsequent movement of a muscle is performed entirely without input from the brain. Even vastly more complicated tasks such as walking and micturition have been shown to rely in part on spinal cord circuitry, with the brain only providing modulation to activate these spinal pattern generators in a purposeful manner (1,2).

As a whole, the spinal cord can be thought of as an extension of the brain, with its own connections, neurons, and autonomous circuitry. This is reflected in the term "central nervous system" (CNS), which entails the brain, the brainstem, and the spinal cord.

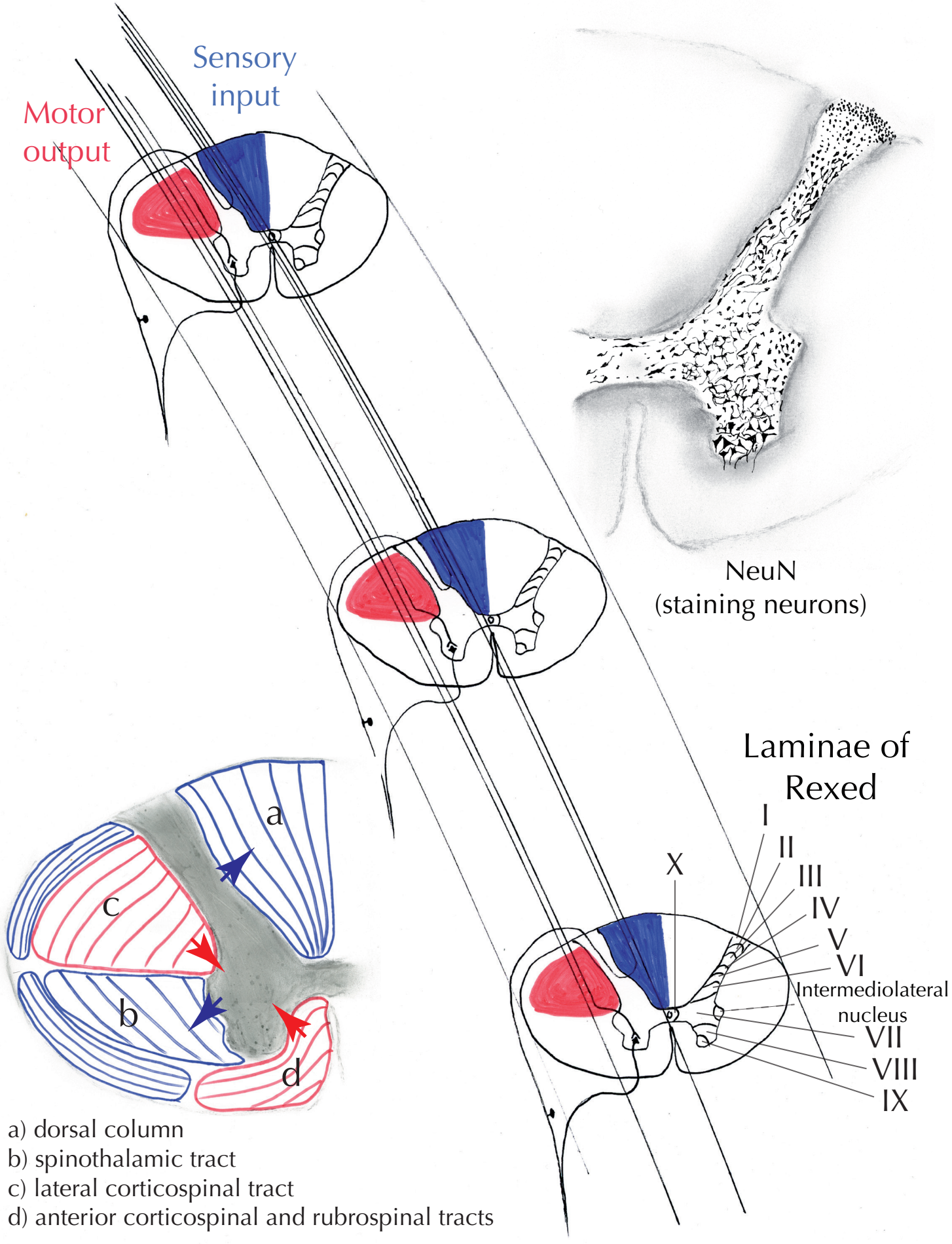
THE BODY IS REPRESENTED IN SPECIFIC SEGMENTS OF THE CORD

The inflow to and outflow from the spinal cord are continuous along the craniocaudal axis ("from head to tail") through dorsal (toward the back) and ventral (toward the front) rootlets. The spinal cord can be anatomically divided into *segments* depending on how these rootlets gather in bundles termed ventral and dorsal roots. The ventral and dorsal roots gather in spinal nerves when they exit the spinal canal.

Outside the spinal canal, the spinal nerves form peripheral nerves or exchange fiber bundles with other spinal nerves in plexae and then form peripheral nerves. The peripheral nerves form connections with muscles and skin in the body and function as cables between the central nervous system and the target organ. Autonomic (non-voluntary) fibers also exit the CNS through the spinal nerves and connect with plexae of the autonomic nervous system, which involves modulation of blood pressure, heart rate, and sweating, among other functions.

The neuronal function of a certain part of the human body is closely correlated to certain segments in the spinal cord, with almost no variation between individuals. Simplified, the arms belong to the cervical spinal cord, the trunk to the thoracic spinal cord, the legs to the lumbar spinal cord, and the anogenital tract to the sacral spinal cord. Because of the anatomy of the spinal cord, a complete injury to the cord at a certain level leads to complete loss of sensory and voluntary motor function below the level of injury.

MICROSCOPIC ANATOMY



a) dorsal column
b) spinothalamic tract
c) lateral corticospinal tract
d) anterior corticospinal and rubrospinal tracts

THE SPINAL CORD IS COMPOSED OF CELL BODIES AND AXONS

The spinal cord tissue is classically subdivided between white and grey matter. White matter is composed of longitudinal axonal connections traversing many segments, often between cortex and spinal cord (corticospinal tracts), brainstem and spinal cord (e.g. rubrospinal tract), or spinal cord and spinal cord (propriospinal tracts). Grey matter contains neuronal cell bodies and shorter axons within the same segment or between adjacent segments (1).

Grey matter can be further subdivided in the lamina of Rexed (1), a classification based on how the cells encountered in the different parts of the grey matter appear in the microscope. Later examination with single-cell transcriptomics (where a more precise identity of the cells are determined by analyzing which genes the cells use) supports the view of a functional laminar organization of the spinal cord grey matter (3,4).

White matter can be classified according to the function of the axonal connection in a specific anatomical location. In animal experiments, the exact location of spinal tracts is well-characterized through the use of neuroanatomical tracers that enable isolation of a specific tract or even single axons (1,5,6). In humans, however, knowledge of the anatomical location of the white matter tracts of the spinal cord is based on careful postmortem dissection, case reports of clinical findings from precisely defined injuries, and post-mortem microscopic examination of patients who suffered cerebral infarction weeks to months before dying of other causes. By observing signs of degeneration of white matter tracts in the spinal cord of these patients, the anatomical location of the projections from the brain to the spinal cord has been deduced (7). Many axons in both white and grey matter are surrounded by myelin sheets electrically insulating the axon more efficiently than cell membranes and enabling faster conduction of the *axon potentials*, the principal long-distance data-transmission mode in the nervous system (8).

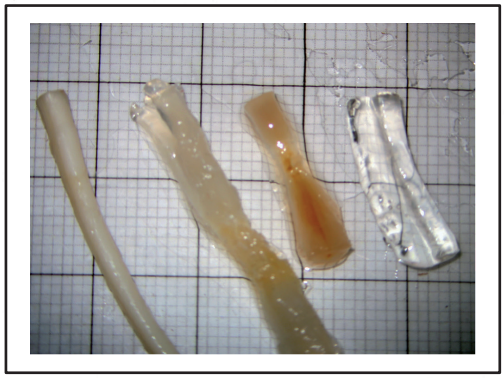
AXONS ARE EXCEPTIONALLY LONG AND THIN

The axonal processes of neurons are exceptionally thin and long. An axon of a couple of micrometers can be one meter long and thus have a width-to-length ratio of 1:200,000. That ratio is comparable to the width of a driving lane from Stockholm to Berlin. An important reason for the lack of knowledge of the exact axonal projections and spinal cord circuitry in humans is that classical wide-field microscopy requires the tissue to be mechanically cut in thin sections. Because of artifacts between sections, reconstruction of intact circuitry has been impossible. A second problem with thin sections is quantification of cell numbers in a tissue volume. This has been solved in the past by various statistical approaches such as manually counting all cells on a defined number of thin sections (9), but the technique has been prohibitively time-consuming for many applications.

OPTICAL SECTIONS PRESERVE THREE-DIMENSIONAL STRUCTURE

Confocal microscopy allows separation of image planes through the optics of the microscope by rejection of out-of-focus light with a pinhole. With optical sectioning, it is possible to image axonal process and cells in much thicker sections than with conventional wide-field microscopy. Unfortunately, mechanical sections are still needed because biological tissue is an inhomogeneous optical medium for visible wavelengths of light resulting in refraction between, for example, extracellular fluid and cell membranes and proteins (10). After passing just half a millimeter through tissue, statistically most photons have changed direction in an uncontrolled fashion at least one time, and extracting high-resolution information is impossible. The effect is similar to shining a flashlight through a finger: the light passes through, but internal structures cannot be visualized because of scattering (10).

OPTICAL SECTIONING



Refractive index
matching of tissue

EXCITATION

DETECTION

TISSUE CAN BE MADE TRANSPARENT BY REFRACTIVE INDEX MATCHING

During the last decade, several methods for homogenizing a refractive index of biological tissue without disrupting structural organization of the tissue on a microscopic and molecular level have been published (11–14). The tissue becomes virtually transparent, and by using microscopic techniques for optical sectioning such as confocal microscopy, questions such as the true course of an axon in the nervous system can be tackled.

The possibility of imaging deep in biological tissue has given rise to a new set of problems. For example, microscopic objectives are commonly made for short working distance between the front lens and the object, creating a physical barrier for imaging deep into transparent tissue. Theoretically, longer working-distance objectives can be built from the exact same design as short working-distance objectives, but scaling up sizes of the glass surfaces greatly increases cost. Second, confocal microscopy records data one voxel at a time, making imaging of three-dimensional volumes impractical because image acquisition time becomes too long. Confocal microscopy also rejects the majority of fluorescence exited, leading to photobleaching in adjacent Z-planes and deterioration of image contrast when fluorescence is used to create image contrast.

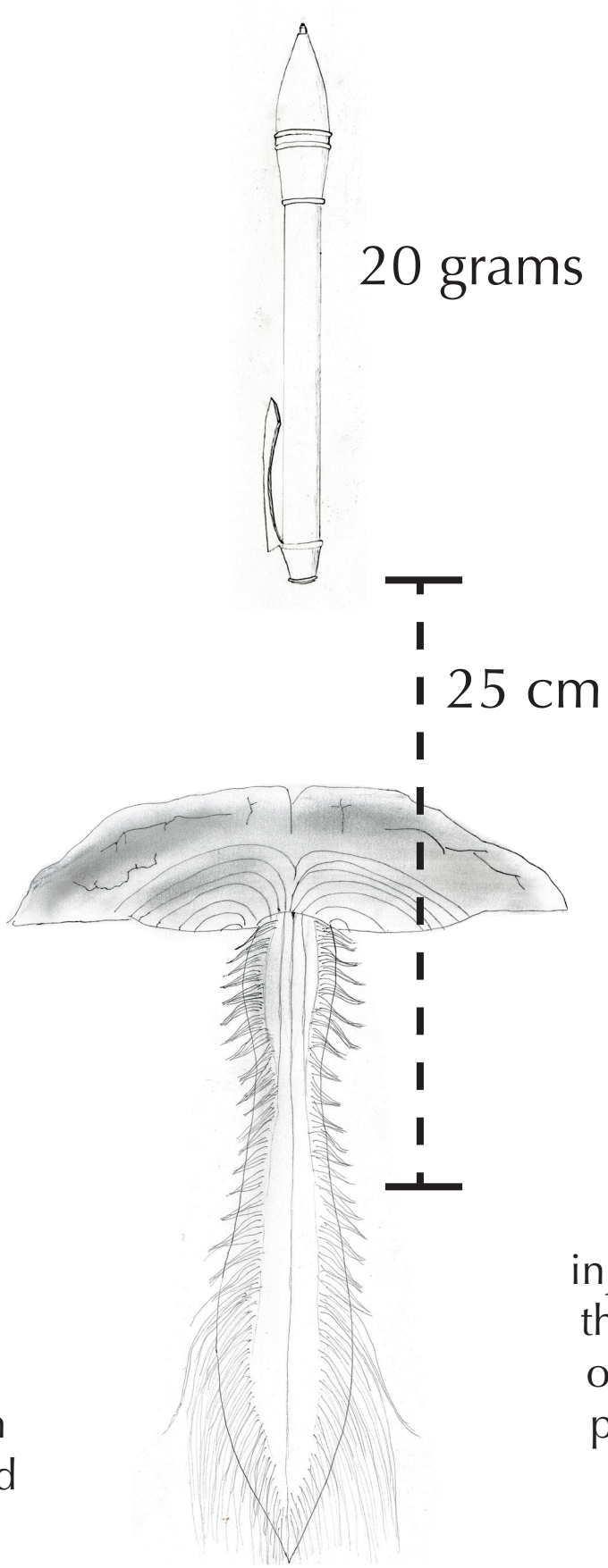
LIGHT-SHEET MICROSCOPY IS IDEAL FOR IMAGING TRANSPARENT TISSUE

Some of the limitations with confocal microscopy for imaging of large volumes of tissue have been overcome by achieving optical sectioning with structured illumination from a second objective situated perpendicular to the detection objective instead of a pinhole. In this setup, wide-field detection can be used, and the image can be captured on a camera sensor (typically millions of voxels per exposure instead of one as in confocal microscopy). Additionally, because only in-focus fluorophores are excited, none of the emitted fluorescence needs to be rejected, leading to reduced photobleaching compared to confocal microscopy (15).

The development of light-sheet microscopes has been rapid in the last decade, with many significant improvements and applications such as multiple light-sheets and detection (16), contrast-enhancing strategies (17), two-photon excitation (18), automatic focusing with adaptive optics (19,20), Bessel-beam illumination (21), transient state imaging (22), super-resolution using stimulated emission depletion (23), or lattice light-sheets (24). Despite the potential of light-sheet microscopy, commercial products are sparse. With low volumes and high prices, they represent a significant investment for most research groups. The different custom light-sheet microscopy setups published are usually aimed at answering different specific questions; therefore, most of the published setups are not directly comparable. Each has its advantage and disadvantage, and the diversity in specifications is significant (25).

Light-sheet microscopy has the potential to gather the information available by making biological tissue transparent and the potential to transform understanding of three-dimensional anatomy of, for example, axonal projections and distributions of specific cell types in normal spinal cord physiology and spinal cord injury. However, for startup projects, the cost of purchasing a commercial light-sheet microscope or establishing a custom instrument is a major barrier to application. An initiative termed "Open-SPIM" has made a significant effort to describe in detail the construction of a cost-effective setup based on commercial optical components (26). However, the price for constructing an Open-SPIM is still around USD 60,000 (27).

THE FRAGILE CNS



The CNS is so soft it will deform under its own weight like a jellyfish when not suspended in water

The CNS could get injured by the the blunt end of a reservoir pen dropped from 25 cm height

INJURY TO THE SPINAL CORD

THE CENTRAL NERVOUS SYSTEM IS SOFT AND FRAGILE

The central nervous system (CNS) is exceptionally soft compared to other tissues in the body. The CNS is "floating" in the cerebrospinal fluid (CSF), and the structural integrity of the CNS is actually so low that it will deform under its own weight when not suspended in water, much like a stranded jellyfish. The CNS is not only soft, but it is also exceptionally vulnerable. Unlike other soft tissues of the body, the CNS does not tolerate fast deformation. On short timescales, the tight-woven complex structure of axons, cell membranes, and small blood vessels in the nervous system behaves almost like glass. The internal structures of the CNS will "shatter" with a surprisingly small deformation. One important reason for this property of the nervous system is the densely packed axons, as showed by recent advances in computer modeling of CNS tissue (28). When deformation of the CNS is slow (e.g. with slow-growing tumors), a remarkable deformation can occur without clinical symptoms. Some authors even propose the elastic modulus of the CNS in this setting to be zero—that is, the CNS is a liquid on long timescales (29–31), just like cats (32).

In a pig model of spinal cord injury (SCI) that resembles human dimensions, a 20-gram weight dropped from a height of 25 cm onto the exposed spinal cord was enough to induce a spinal cord injury (33). The force used in the study is equivalent to dropping the blunt end of a reservoir pen from your raised hand onto the desk in front of you. Dropping it on your other hand or any other part of the body would induce temporary discomfort but not even result in a bruise. The exposed spinal cord, however, would receive an injury with a possibly life-long deficit of function.

The CNS is also vulnerable to ischemia. After just a couple of minutes of anoxia, cellular physiology deteriorates to the point of irreparable damage to the tissue. Because traumatic deformation of the spinal cord commonly leads to microvascular injury, some of the detrimental effects of trauma to the spinal cord will be caused by ischemia alone (34). Normal physiological arterial pressure in humans can also be enough to cause structural damage to the tissue in the case of rupture of an artery in the CNS (e.g. in subarachnoid hemorrhage) (35).

The vertebrae and ligaments surrounding the spinal cord protect the soft and vulnerable neural tissue from injury in most situations encountered in daily life. The structure of the spine represents a developmental trade-off between low weight and flexibility and protection of the neural structures. When studied in the laboratory, the breaking point for normal vertebrae for axial pressure is 3-4 kN, roughly 3-400 kg placed on top of the head in normal gravity (36,37). Real-life injuries are more complicated, with various angles of force and rotational or penetrating trauma, possibly lowering the force needed for injury.

Most traumatic spinal cord injuries are the result of transport-related accidents and falls (38). The mechanism of injury supplies the forces needed to exceed the breaking point of many structures in the human body, including the spine. Injury to the spinal cord itself is often inevitable in trauma leading to an unstable vertebral fracture because the delicate neuronal tissue will suffer injury immediately if the spinal canal is compromised by surrounding structures.

SPINAL CORD INJURY IS A MEDICAL EMERGENCY

Because of the forces needed to injure the vertebral column to the point at which spinal cord injury occurs, traumatic spinal cord injury (SCI) is commonly part of multi-trauma. Therefore, in the immediate pre-hospital or emergency room setting, life-saving interventions are always prioritized such as optimizing airway, breathing, and circulation (39). A severe SCI high enough in the cervical cord (above C_3) will require artificial ventilation within minutes after the injury for the patient to survive, and the patient will be dependent on ventilator support throughout life. However, for any

ACUTE SCI

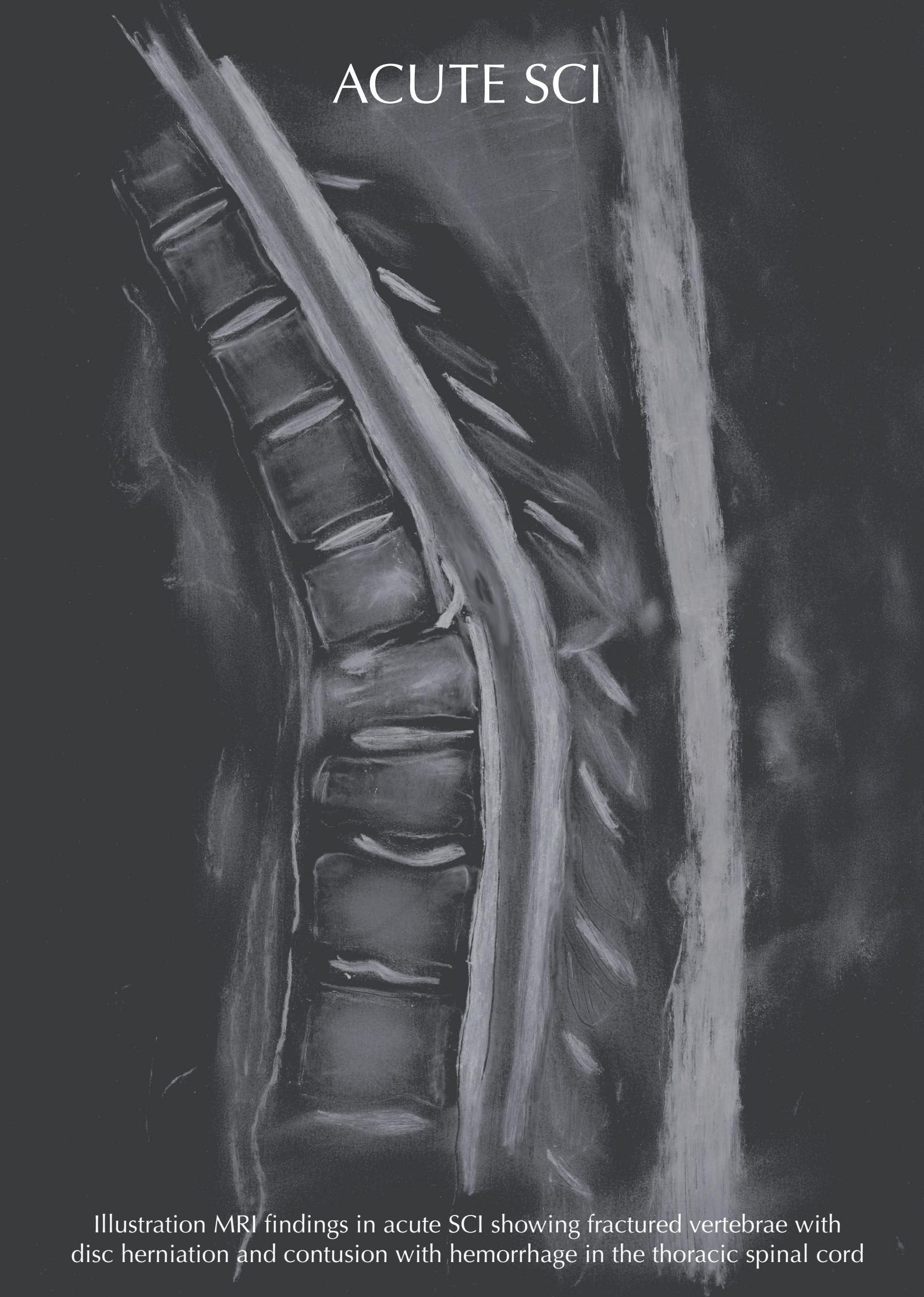


Illustration MRI findings in acute SCI showing fractured vertebrae with disc herniation and contusion with hemorrhage in the thoracic spinal cord

patient in whom vertebral column injury cannot be excluded, great care must be taken to avoid inducing or worsening neurological damage by changing the anatomy of the fracture by mobilization. Therefore, all trauma patients should be put on a spine board to immobilize the spine until injury has been ruled out (40).

In most clinical guidelines for severe trauma, patients undergo computerized tomography (CT) of the entire vertebral column at an early stage. CT testing has high sensitivity to vertebral fractures, catching the majority of suspected spinal cord injuries even in unconscious patients. Plain X-ray can miss some vertebral fractures and is not recommended (41).

A small portion of spinal cord injuries occur without injury to the vertebral column and with injury to spinal ligaments and spinal cord alone (2 out of 45 in a recent Stockholm cohort) (42). If such a patient is unconscious from concomitant injuries, an MRI would be needed to find the injury. Therefore, in unconscious trauma patients for whom intubation is required, fiber optic intubation without the need for mobilization of the patient's neck is recommended (43).

If a spinal cord injury is confirmed or suspected, an early MRI should be performed. Advantages of early MRI include a definite diagnosis as well as the possibility for outcome prediction based on the MRI findings (44). MRI can also indicate whether the spinal cord is under pressure from surrounding structures or hematoma and provide clarity for early decompressive surgery.

If the spinal cord is under pressure from surrounding structures, preclinical evidence strongly suggests and clinical studies indicate that early decompressive surgery improves neurological outcomes but also decreases the risk for other complications. Also, avoiding hypotension and hypoxia improves outcome in preclinical studies and is being tested in a clinical trial (40).

Methylprednisolone is often given in an initial high-bolus dose (30 mg/kg) and then a continuous infusion for 24 hours (5.4 mg/kg/hour), despite contradictory evidence (45–47).

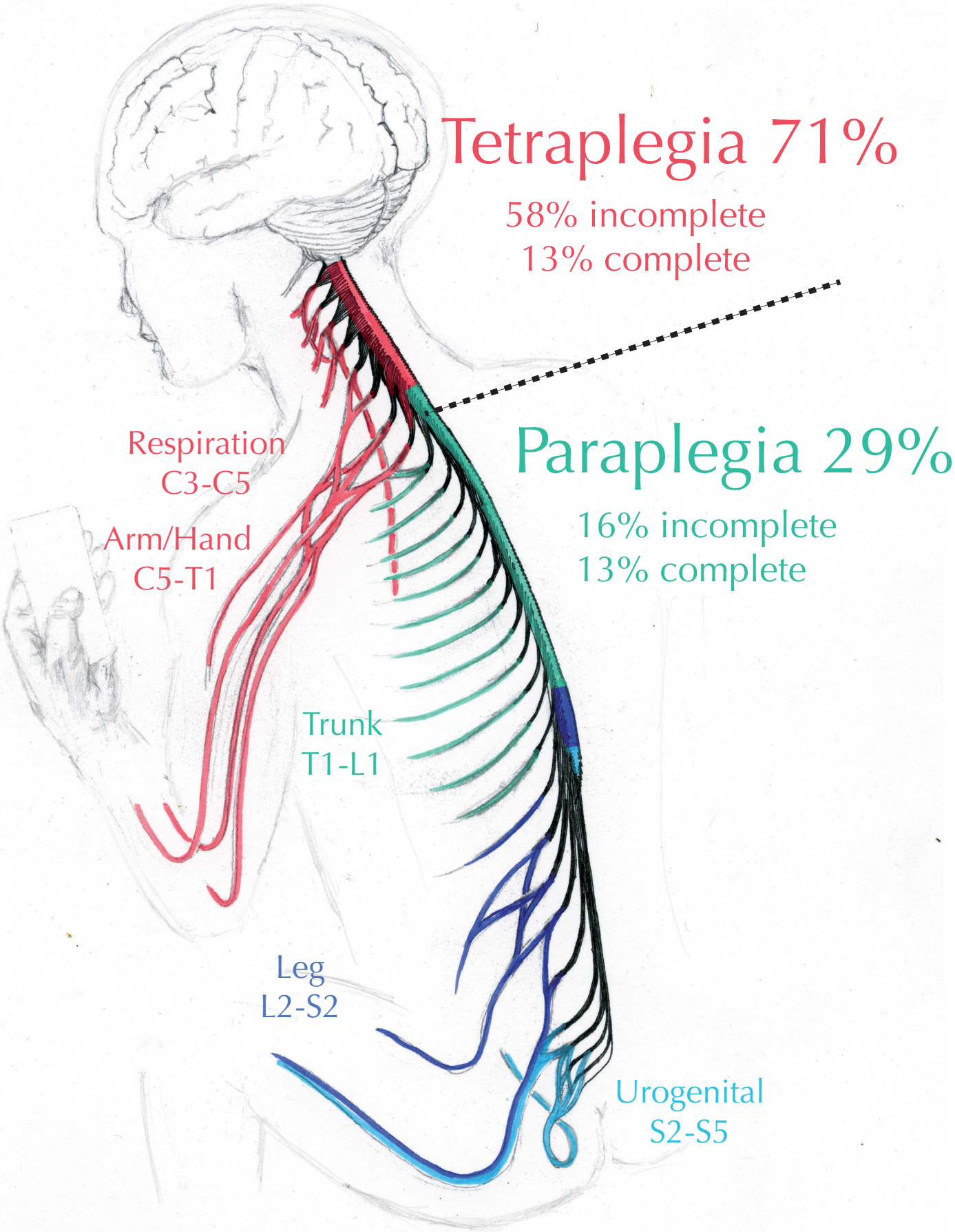
Acute SCI represents a significant challenge for the health care system. In low-income settings, in-hospital mortality after SCI can be as high as 35%; in high-income regions, in-hospital mortality is around 8% (48). At present, early surgery for decompression with support and stabilization of vital parameters are the only truly evidence-based interventions in acute spinal cord injury.

SPINAL CORD INJURY IS CLASSIFIED BASED ON LEVEL AND SEVERITY

Spinal cord injury in a patient is classified based on segmental level of injury and severity of injury. Injury to the cervical spinal cord affecting both legs and arms is called tetraplegia, and injury to the thoracic or lumbar spinal cord affecting the legs is called paraplegia. Complete injuries show no voluntary function or sensation below the neurological level of injury, and incomplete injuries have varying degrees of function spared.

For more precise classification of spinal cord injury, most health care systems (including the Swedish) use the American Spinal Injury Association (ASIA) and International Spinal Cord Society (ISCOS) International Standards for Classification of Spinal Cord Injury (ISNCSCI) assessment form *(asia-spinalinjury.org/wp-content/uploads/2016/02/International_Stds_Diagram_Worksheet.pdf).* The ISNCSCI form and published instructions detail the testing and classification of patients with spinal cord injury in a structured manner (49). The form defines the segmental sensory level by light touch and pinprick and the motor level by grading of strength. Each segment tested renders a point score, adding to the sensory score and motor score. The neurological level of injury is defined as the most caudal (toward the tail) neurological segment showing normal sensation and antigravity muscle strength. Further, completeness of the injury is tested by evaluating sacral sparing. If no voluntary anal contraction and no sensation to deep anal pressure are present, the injury is termed complete. Thereafter, the specific ASIA Impairment Scale (AIS) level is determined.

CLASSIFICATION OF INJURY



The AIS-levels are defined as follows:

AIS-A encompasses sensorimotor complete injuries, defined as no sacral sparing and no residual muscle function more than three segments below the neurological level of injury.

AIS-B encompasses sensory incomplete injuries showing sacral sparing of sensation but no voluntary anal contraction and no residual motor function more than three levels below the neurological level of injury.

AIS-C encompasses motor incomplete injuries showing voluntary anal contraction and sensation or sensation to deep anal pressure and residual muscle function more than three levels below the neurological level of injury.

AIS-D encompasses motor incomplete injuries by the same definition as AIS-C but with significant residual motor function in which more than half of the muscles below the neurological level of injury have antigravity muscle strength.

AIS-E encompasses patients with a history of spinal cord injury and previous higher AIS classification but normal sensory and motor scores on examination with the ISNCSCI assessment form.

The ISNCSCI assessment form gives clinicians and researchers all over the world a common language of classification for patients with spinal cord injury and enables patient stratification and observation of improvement or deterioration in neurological function.

In the initial days after an SCI, the patient often presents with total areflexia below the injury level, referred to as *spinal chock*, which gradually transforms to spastic paresis with varying degrees of severity and level of spasticity (50). Because of the spinal chock, AIS-level scoring at three days or more after injury has higher predictive value for long-term AIS level than if assessed immediately after injury (51). Many patients experience improvements in function and even transitions in AIS grade months after injury, and such improvements have been recorded more than 12 months after injury, albeit rarely (51).

INCOMPLETE CERVICAL SCI IS THE MOST PREVALENT INJURY TYPE

A study performed prospectively in the greater Stockholm area from 2014-2015 identified 49 cases of SCI during an 18-month period and calculated crude incidence rates of 19.5 per million per year (42), which is consistent with other reports in the literature from regions with similar socioeconomic and demographic features (52). The median age in the Stockholm cohort was 58 years (range 18-85), and most injuries resulted in tetraplegia (32/45), with the majority of injuries being incomplete (25/32, AIS B-D). Of the injuries resulting in paraplegia, 6/13 were complete (AIS A).

Almost all of the 45/49 injuries included in further analysis (44/45) from the Stockholm cohort were caused by transport-related events (18/45) and falls (26/45). Of the transport-related injuries, the most common causes were motorcycle accident (9/18) and bicycle accident (7/18). Among the falls, the authors reported an interesting stratification by age: 8/10 falls in the age group under 60 years were from higher than three meters, whereas 12/14 falls in the age group over 60 years were same-level falls from under one meter (42).

CHRONIC SCI

Illustration of MRI findings in the chronic stage after SCI showing status after laminectomy and a post-traumatic cyst in the thoracic spinal cord

Compared to a similar study in the Stockholm region in 2006-2007, the injury panorama for SCI has drifted, with a decrease in sports injuries and an increase in older women suffering incomplete cervical injuries from same-level falls (42,53).

A recently published study utilizing data from the Global Burden of Disease (GBD) project—in which data from 195 countries are continuously gathered and made available for researchers around the world—estimated that 0.9 million spinal cord injuries occurred worldwide in 2016, and 27 million people were living with chronic spinal cord injury worldwide at that time (38).

The same study calculated that the age-standardized incidence was 13 per year per 100,000 people with a prevalence of 368 per 100,000 globally. In Sweden, as well as other high-income countries, spinal cord injury was more common according to the study: the age-standardized incidence was 26 per year per 100,000 people, and the prevalence was 903 per 100,000 people. The study estimated that 101,000 people are living with spinal cord injury in Sweden. These figures are inflated by a factor of 10 compared to the crude incidence rates reported by Joseph et al. (42) and by about the same amount when compared to other published reports of the demographics of SCI (38). The reason for this difference in incidence when using these separate methodologies is unclear and under investigation *(personal communication with the author).*

REHABILITATION AND SPECIALIZED CARE ARE VITAL AFTER INJURY

After the patient is medically stable, rehabilitation should start as soon as possible to prevent secondary complications and maximize function following injury. Rehabilitation has been shown in experimental studies to be vital for inducing plasticity and increasing recovery of neurological function after SCI in e.g. rats (54). In humans, high-level evidence of the positive effect of rehabilitation on neurological outcome is lacking, but the association is universally accepted (55). Rehabilitation also serves many other objectives such as improving the patient's independence in activities of daily living and identifying and treating secondary complications. Recent studies on rehabilitation generally focus on type and timing of rehabilitation, but the level of evidence for a specific strategy is currently low (55). An emerging concept is robotic-assisted rehabilitation, by which the movements if rehabilitation are specifically controlled and supported to be physiologically beneficial (56,57). This strategy has yet to be proven to be superior to conventional rehabilitation with a physiotherapist, but it has the advantage of being standardized and the possibility of measuring improvements in areas such as weight-bearing more precisely.

The enormous improvements seen in survival of patients with SCI during the 2000th century (58) is most certainly not only attributed to medical technology such as antibiotics but also to the establishment of specialized rehabilitation units actively following patients and treating complications preemptively. The following section describes some of the complications commonly seen in chronic SCI.

BLADDER PROBLEMS ARE COMMON AND CHALLENGING

In the period of spinal shock, the urinary bladder is commonly paretic, and severe urinary retention ensues if the bladder is not emptied. If not treated, increased pressure can result in kidney damage. With time, varying degrees of reflex micturition, leaking bladder, or normal urinary function appear. A higher risk for urinary tract infection and urosepsis prevails in the SCI population throughout life. Clean intermittent catheterization is commonly used for bladder management because it greatly reduces the risk for infection compared to indwelling catheters (59). Bladder management is an absolute necessary intervention. In an older study from the UK from 1992, urinary tract infection was the leading cause of death in the population with chronic SCI (24.3%), even higher than for

cardiovascular disease (23.2%) (60). These numbers were greatly reduced during the 1990s and 2000s to 6-8% (61).

Bowel function is also impaired after injury and can cause both constipation and fecal incontinence but usually does not result in the sort of life-threatening complications seen with bladder dysfunction (62).

SEXUAL FUNCTION IS PRESERVED BUT OFTEN ALTERED

The overall impact on sexual function after SCI is dependent on the location and severity of the injury, as well as on other SCI-related complications such as incontinence, spasticity, or pain (63,64). Subjective arousal can be experienced by SCI patients independently of the level of injury (65)m=, whereas (reflexive) genital responses such as lubrication in women and erection in men is commonly affected to some degree (64). In sacral injuries, it can be absent; in higher injuries, a reflex response is usually present (64). Erection in men can be supported with common medications for erectile dysfunction, and this intervention has been shown to increase HRQoL (66,67). The sexual act itself is also possible to conduct independent of the SCI but might need adaptations in order to be compatible with the limitations caused by the injury. For the partner of a SCI patient, this might involve modifications of previous sexual routines. Orgasms are commonly absent or different compared to pre-injury but are more often preserved among female patients. This is true even in complete injuries, possibly due to vagal innervation of the cervix (68,69). In a study of all Scandinavian community-living women with SCI, only 8% did not experience orgasms post-injury (70). In men with SCI, ejaculation is often not possible, or retrograde ejaculation occurs because of a lack of coordination after injury, with anejaculatory infertility as consequence (71). Ejaculation can, however, be elicited in more than 50% of men with complete injuries using vibratory stimulation of the penis and perianal area (72,73). Semen can then be retrieved for IVF, and direct intravaginal insemination in the home setting has led to pregnancies in a significant number of cases (71).

In summary, sex continues to be an important aspect of life after SCI (64). Yet, the sexual experience is often altered, and the SCI patient and his or her partner need to develop a new sexual routine. Importantly, sex for reproductive purposes is often possible.

PRESSURE ULCERS ARE POSSIBLE TO PREVENT

Pressure ulcers are another common complication of SCI because of immobility and loss of sensibility. Although common, it is completely preventable both short- and long-term, as shown more than 50 years ago in an SCI center in the UK (74). Recent advances include electronic pressure sensing and feedback to the patient that adjustment of position is warranted to avoid pressure ulcers (75,76).

SPASTICITY CAN HAVE BOTH POSITIVE AND NEGATIVE EFFECTS

After the loss of supraspinal input to the spinal cord circuitry, an increased reflex activation of spinal motor neurons commonly occurs after SCI and other events such as stroke. This leads to the clinical manifestation of spasticity where the paretic body part produces involuntary movement, often in response to a sensory stimulus (77). The spastic muscles after SCI show increased tonus and a change in fiber composition (78).

Clinical measurements of spasticity in SCI are negatively associated with Health Related Quality of Life (HRQoL) because it can interfere with movement and social functioning (79), but they are also positively associated with muscle mass below the injury level (80). Although mostly negative for the patient, spasticity can also be positive when a higher tonus of musculature leads to

findings like improved trunk stability, better weight-bearing for stepping in incomplete injuries, or stronger reflex genital response (77).

NEUROPATIC PAIN IS COMMON AND DIFFICULT TO TREAT

Pain is a common problem after SCI. Pain can arise for a number of different reasons such as immobility or bad seating in a wheelchair, but the most challenging is neuropathic pain. A Swedish study found a prevalence of neuropathic pain of 40% in spinal cord-injured patients of all levels and severity (81), and these figures are in line with other reports from the literature (82). The strongest predictor of neuropathic pain in the Swedish study was older age, and 70% of patients experiencing neuropathic pain stated that the pain was a problem in their daily life (81).

Neuropathic pain can arise in a variety of conditions in the nervous system such as stroke, Parkinson's disease, and peripheral nerve injury. Some form of lesions in sensory pathways is considered necessary, and neuropathic pain is usually not encountered where sensation is normal (83). One study in spinal cord-injured patients showed that neuropathic pain was most prevalent in patients with small residual spinothalamic function (84).

Management of neuropathic pain can be challenging because many common pain medications are ineffective, and some have problematic long-term effects (83). A recent Canadian consensus (85) developing clinical guidelines for the management of neuropathic pain in SCI suggests using the International Spinal Cord Injury Pain Basic Data Set (86) for classification and follow-up of neuropathic pain. The same consensus group published detailed clinical guidelines for treatment, advocating first-line medical treatment as follows: first pregabalin, thereafter gabapentin, and then amitriptyline (82).

AUTONOMIC DYSREFLEXIA CAN BE DANGEROUS

SCI patients with a neurological level of injury above the midthoracic level (commonly cited as injuries above T6) show a varying tendency to develop autonomic dysreflexia. The condition is most often seen in tetraplegic patients, and is a potentially life-threatening condition characterized by a sharp increase in blood pressure well over 200 mmHg (87). Normal systolic blood pressure in this patient group is commonly under 100 mmHg (87). It has been reported to result in seizures, cerebral hemorrhage, and sudden death (88–90). Autonomic dysreflexia is thought to be triggered by noxious stimuli or an imbalance in the paretic part of the body such as bladder distension (87,90), but the precise pathophysiological mechanism is still debated (91). Blood pressure measurements of SCI patients have shown that sharp variations of blood pressure are much more common than the episodes of severe hypertension noted by patients and health care professionals, and they are implicated as an explanation for the increased cardiovascular risk seen in some groups of SCI patients (92). Therefore, novel methods for measuring and detecting the blood pressure variations seen in SCI are under investigation (93).

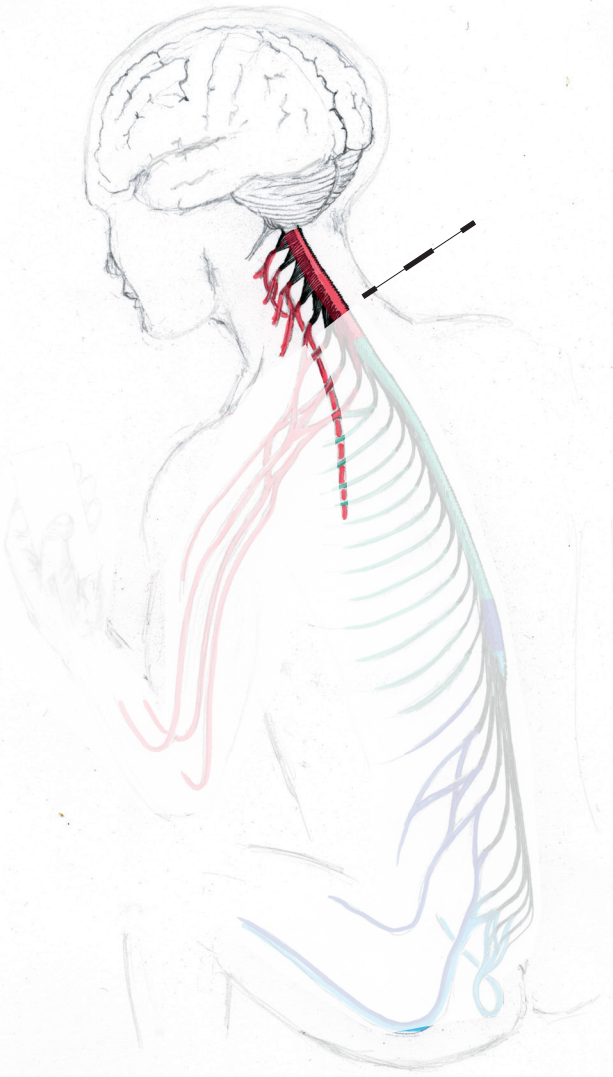
LIVING WITH CHRONIC SPINAL CORD INJURY

Defining the impact of SCI on an individual can be done in many different ways such as describing the neurological function in the patient (49), analyzing mortality (58), and tracking the incidence of medical complications such as urinary tract infections (94), fertility (71,95), years lost to disability (38), and employment status (96).

After the acute phase, mortality rates in the SCI population continue to be increased (48), but life expectancy in chronic SCI varies strongly with injury level and severity. In AIS-D, life expectancy is the equal to that in the normal population. In AIS A-C, high cervical injuries (C1-C4) have about

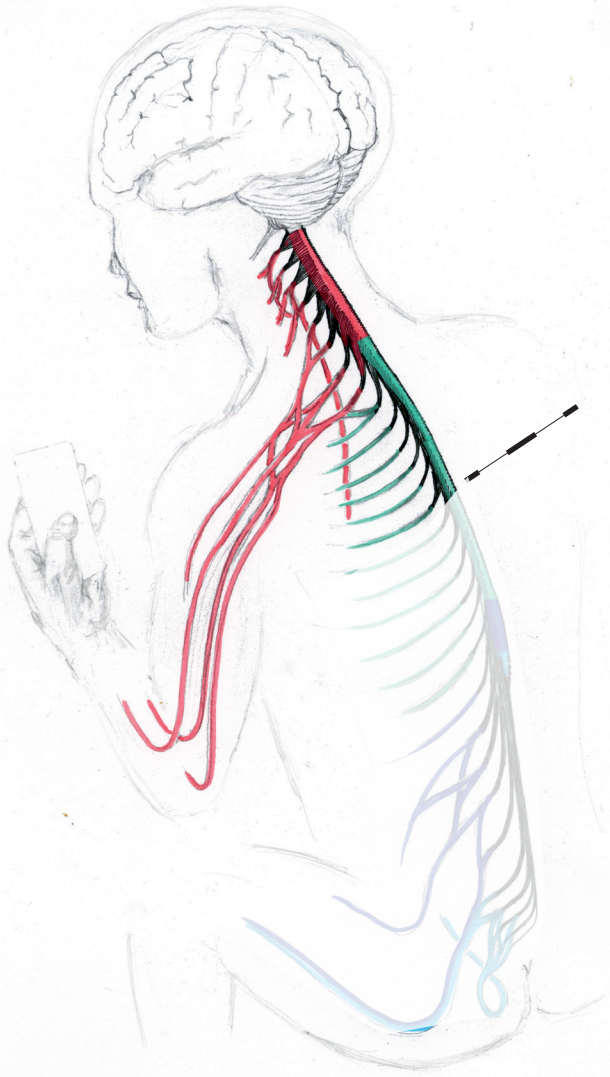
PRIORITIES

Tetraplegia



Arm/hand function 49%
Sexual function 13%
Trunk stability 11%
Bladder/bowel 9%
Walking 8%
Sensation 6%
Pain 4%

Paraplegia



Sexual function 27 %
Bladder/bowel 18%
Trunk stability 16%
Walking 16%
Pain 12%
Sensation 7%

After Andersson 2004

65%, low cervical (C5-C8) have 70%, and T1-S5 have 90% of the life expectancy of the normal population (97). Respiratory infection was the most common cause of death (29.2%) from 2004-2015 in a cohort of chronic SCI patients studied in the UK, followed by cardiovascular (21.8%), neoplasms (15.9%), and urogenital disease (8.7%) (61). Depression is more prevalent throughout life after SCI than in the normal population (98), and the Global Burden of Disease recently estimated that years lost to disability are similar in SCI as in traumatic brain injury, despite traumatic brain injury having a 30-times higher incidence rate (38).

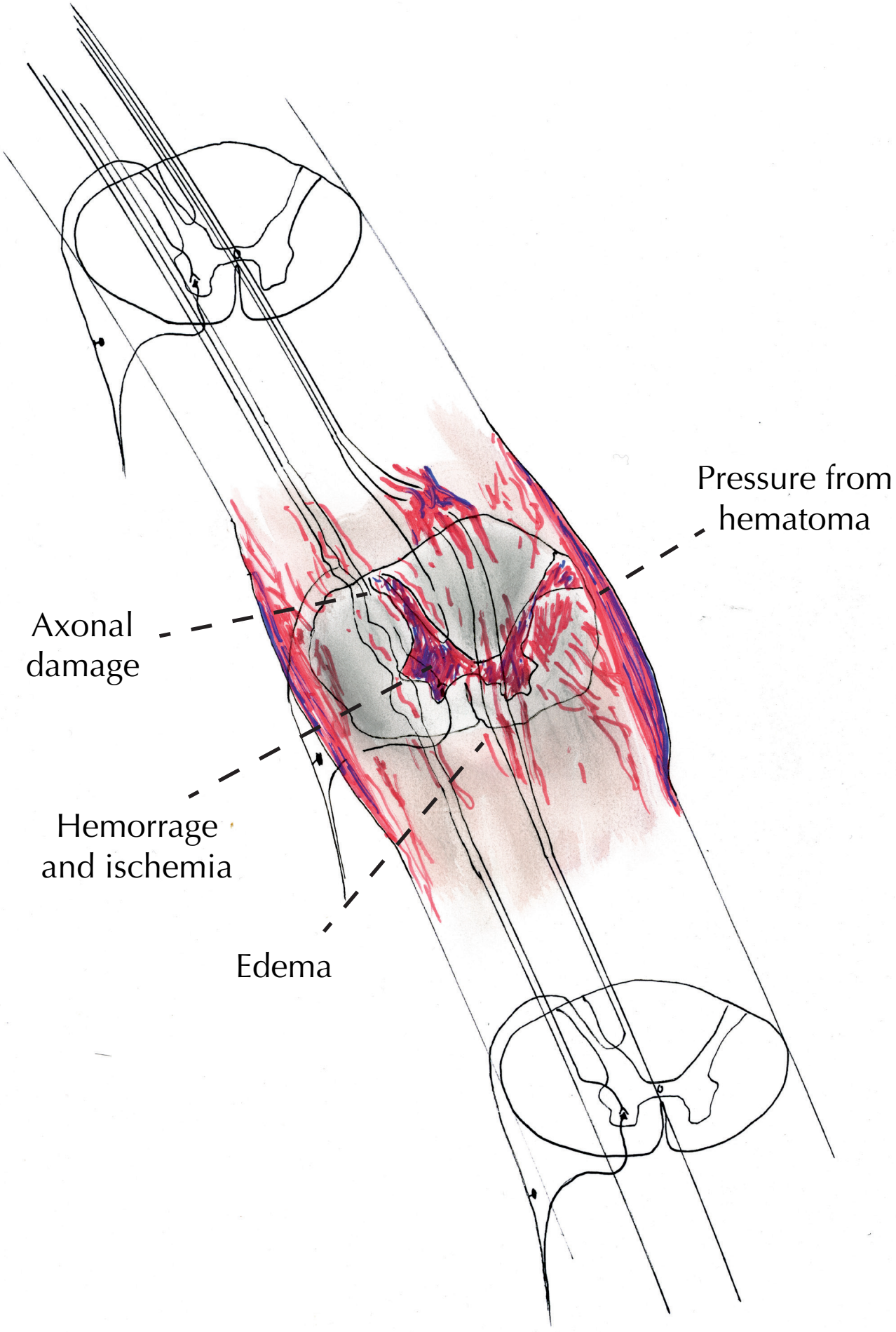
An important aspect of SCI not covered by these measures is the subjective experience of living with SCI and Health-Related Quality of Life (HRQoL). Subjective experience can be analyzed by measures such as qualitative interview-based methods and give fundamental insight into important aspects of human experience and behavior (99). Although an important strategy, findings across studies—even within the same patient over time—can be hard to compare in a quantitative fashion. Structured or semi-structured instruments for HRQoL such as the EQ-5D or more extensive instruments can overcome this problem and have been used in SCI (100,101) and many other neurological conditions (102).

Measurements of HRQoL consistently show a decrease after SCI, often in connection to medical problems (94,100,103,104). To account for and measure adequate health-related quality of life data in the SCI population more efficiently, a recent effort (called SCI-QOL) has created specific but adaptive questionnaires with the ability to measure quality of life at the current level of functioning in an SCI patient (105). This approach has an advantage over instruments developed for the general population because it captures specific issues after SCI in greater detail and avoids questions that are irrelevant or even offending (105). Unfortunately, it has not yet been translated and validated in the Swedish language.

The priorities of individuals living with chronic SCI have also been studied: for tetraplegic patients, regaining arm function is the highest priority, whereas for paraplegic patients, sexual function is the highest priority. Bladder and bowel control rank high for both paraplegic and tetraplegic patients. Thus, contrary to popular belief, walking is not the highest priority in the SCI population and often ranks third or fourth place (106). A meta-analysis of priorities largely reflected the result from the study by Anderson in 2004, but emphasize the impact of how questions are asked, and the injury level and severity in the patient group answering the question (107).

In conclusion, spinal cord medicine has improved greatly during the last decades, boosting life expectancy and the arsenal of battling chronic medical problems after SCI, although similar goals apply today, as stated more than 50 years ago by one of the early pioneers of spinal cord injury medicine Sir Ludwig Guttman, preventing common medical problems and supporting the patient through physical and occupational rehabilitation to a meaningful and productive life (74).

ACUTE INJURY



BIOLOGICAL MECHANISM AND CLINICAL TRIALS IN SCI

PRIMARY INJURY LEADS TO IMMEDIATE LOSS OF FUNCTIONS

As described in an earlier section, the tissue of the spinal cord is both soft and brittle and is injured by a small mechanical insult. Shattering of the complex organization of the neural tissue leads to loss of axonal connections and cell rupture at the very moment of the injury. As a result of microvascular injury, disruption of oxygen and nutrient supply quickly expands cell death beyond the momentary loss of structures. In experimental injury, cell death in both neurons and glial cells begins immediately after contusion injury and levels out after about four hours (108).

In clinical SCI, the initial impact is often followed by delayed pressure due to displaced surrounding tissue, swelling of the injured spinal cord, or hematoma (109). Experimental studies in animals have shown that delayed pressure to the cord results in worse neurological outcome by a factor of time and pressure and that early decompression can rescue the negative effect (110). Several publications on clinical SCI cases have shown clinical benefit in terms of long-term neurological outcome in patients undergoing early decompressive surgery, with recent definitions of "early" being within eight hours after trauma (111–113), but to date no randomized trial has been completed (114).

Increasing spinal cord blood flow by elevation of mean arterial pressure and cerebrospinal fluid drainage has been shown to improve outcomes in experimental SCI in the pig (115). A phase I/II clinical trial showed safety but failed to show efficacy due to lack of power (116); a phase II/III study is ongoing (NCT02495545).

Lowering tissue demand of oxygen and nutrients by hypothermia has been suggested for many conditions including acute SCI. Modest hypothermia (32-34°C) has been shown to be safe (117), and a randomized clinical trial has been planned but not yet performed (118).

Measurement of biomarkers in serum and CSF after traumatic brain injury have led to important advances in clinical management and outcome prediction (119,120). Recently, a set of biomarkers collected from CSF drainage (when studying the effect of CSF drainage mentioned above) was shown to predict AIS grade after injury, even surpassing the predictive power of MRI (121).

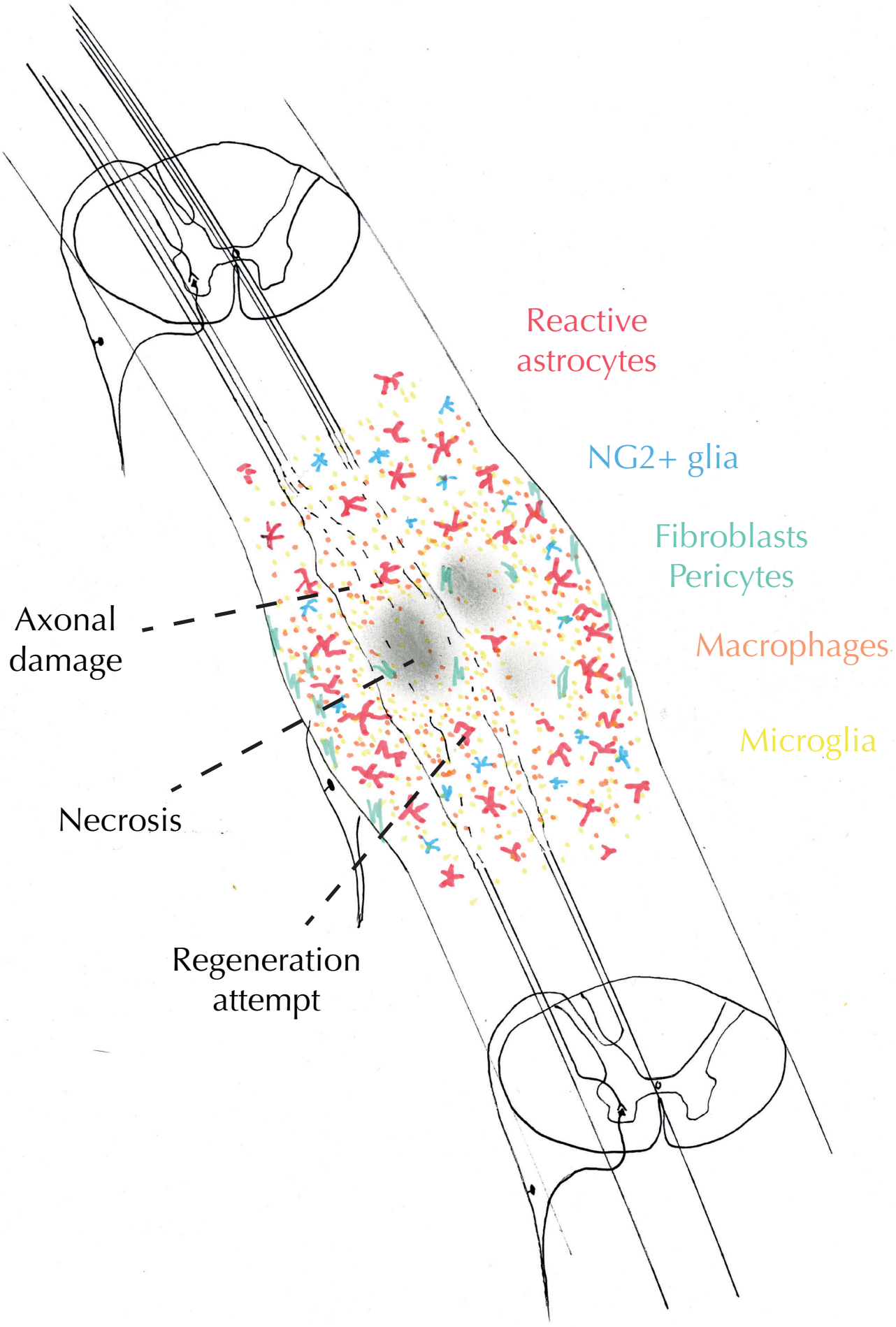
Prevention of further ischemic injury, delayed primary injury by early decompressive surgery, and prevention of hypoxia and hypotension are the only current medical interventions for ameliorating the primary injury at present, and hypothermia is a potential future acute intervention if efficacy can be proven (118). Apart from this, primary prevention strategies in society are the only feasible interventions for battling primary injuries because of the short time frame from physical insult to manifest injury.

CNS WOUND HEALING RESULTS IN SECONDARY INJURY AND A GLIAL SCAR

Mechanical injury to cell membranes and microvasculature with necrotic cell death and disruption of the blood-brain barrier starts a cascade of events, with important functions for preventing infections, clearing tissue debris, re-establishing the blood-brain barrier, and re-establishing tissue homeostasis (122). After the acute physical insult resulting in an SCI (or injury to the CNS in general), there is clear evidence of a secondary injury in which further loss of neurons, axons, and myelin occurs (118,122,123). This phase last hours to weeks after injury and is characterized by activation of the immune system; inflammation; proliferation of glial cells, endogenous stem cells (124) and pericytes (125), and upregulation of extracellular matrix proteins. The secondary injury cascade matures into what is commonly termed "the glial scar" (126,127).

Immediately after injury, disruption of the blood-brain barrier, hemorrhage, and apoptosis trigger microglia proliferation as well as infiltration of blood-derived neutrophils and macrophages.

SUBACUTE PHASE



Immune cells protect the tissue from infection in non-sterile wounds, and phagocytizing cells clean debris and necrotic tissue and stimulate angiogenesis in the injury zone (122). The immune reaction also produces a powerful inflammatory response through release of pro-inflammatory cytokines including IL-1β and TNF-α in the tissue with further damage to neurons and glia (122). In the early phase, blood derived macrophages rather than microglia has been suggested as the main culprit in the secondary dieback of axons rather than resident microglia (128). Microglial response has sometimes been attributed to a pro-inflammatory cytotoxic phenotype (M1) and an alternative activation (M2), but inconsistent findings and single-cell RNA sequencing have resulted in a vastly more complex understanding of microglial phenotypes both in normal physiology and in injury (129).

The initial inflammatory response to injury triggers proliferation and migration of glial cells into the lesion via mainly chemokine signaling (127). Astrocytes and cells positive for the marker NG-2 have been studied extensively as they proliferate and differentiate (130). One study showed that the initial astrocyte response supported axon regeneration during the first two weeks after SCI in mice, but further exposure of the astrocytes to mainly collagen-1 (usually not present in the CNS) transformed astrocyte response into a scar-forming phenotype that inhibited regrowth of axons (131). Abolishing the astrocyte response was also associated with worse neurological outcome in mice (132). Another recent advance in the understanding of astrocytic response to injury in a shorter time frame after injury is the identification of the neurotoxic A1 subtype activated by reactive microglia through the cytokines Il-1α, TNF, and C1q (133,134). A1 astrocytes, in contrast to the A2 phenotype, were shown to lose many normal astrocytic functions and induced death of neurons and oligodendrocyte *in vitro*.

In mouse contusion SCI, NG-2-positive cells of oligodendrocyte origin differentiate and form 25% of the astrocytes in the glial scar (135). Another NG-2-positive cell (type A pericytes) has also been shown to proliferate extensively after injury and promote physical closing of a spinal cord injury lesion (125). Abolishing the pericyte response led to worse functional outcome after injury (125), but a balanced reduction of the response promoted recovery in mice after spinal cord injury (136).

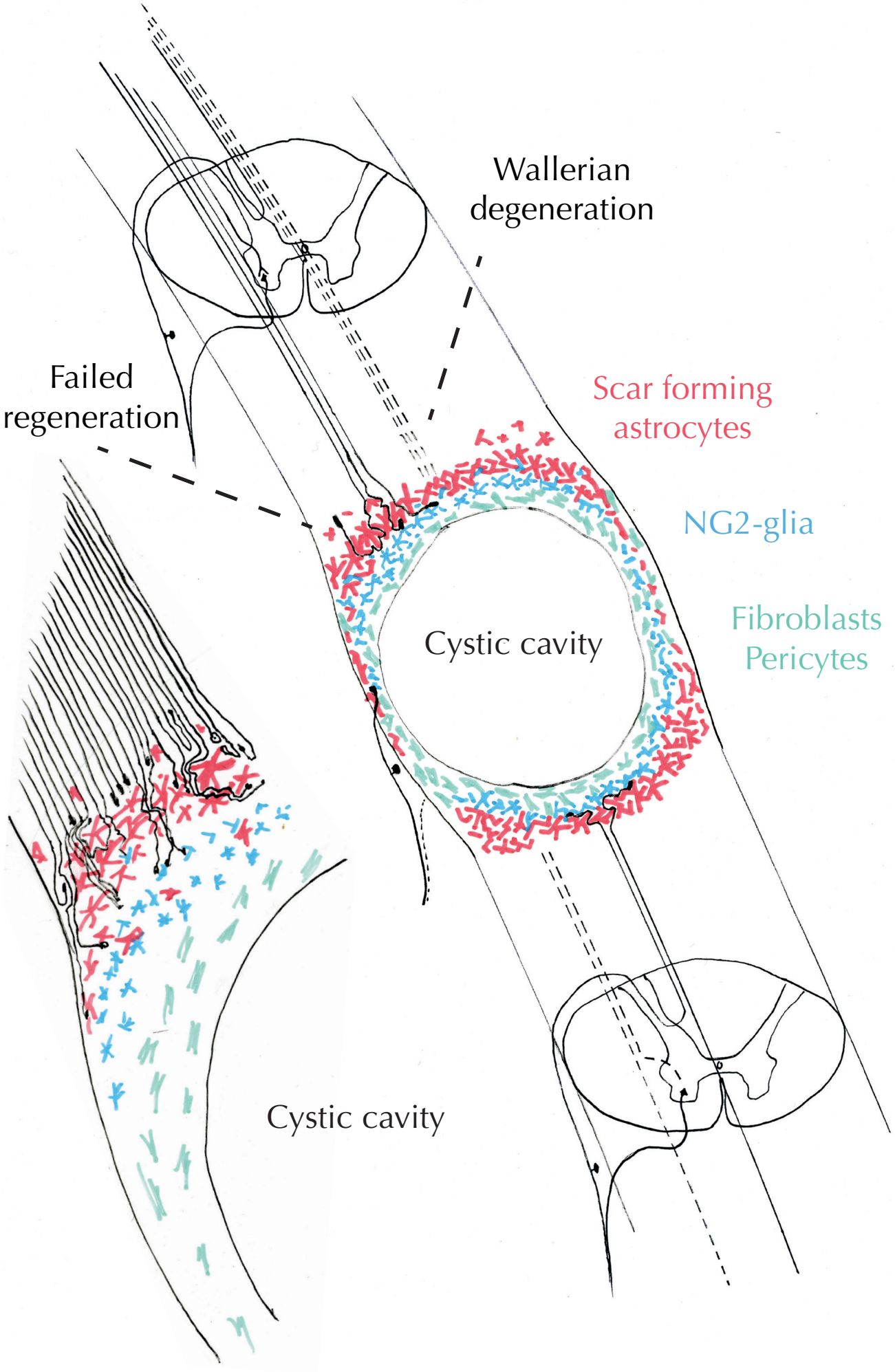
During the first weeks after injury, the proliferation of astrocytes, oligodendrocyte progenitors, and pericytes leads to marked increase of production of a class of extracellular matrix proteins called chondroitin sulphate proteoglycans (CSPGs) (126). These have been shown to play a major role in inhibition of axonal outgrowth and the enzymatic breakdown of CSPGs by application of the bacterial enzyme chondroitinase, which is associated with recovery of function after injury (137,138). Another important inhibitor of axon outgrowth after injury is the myelin-associated neurite outgrowth inhibitor termed NOGO (139).

CLINICAL TRIALS TARGETING SECONDARY INJURY AND GLIAL SCARRING

For good reasons, the majority of interventional clinical trials in SCI have focused on the subacute phase in order to minimize secondary injury. Despite preclinical success, all pharmaceutical interventions that have completed phase III to date have failed to show efficacy for the primary endpoint. Some promising approaches are in earlier phases of clinical trial or in ongoing phase III.

Methylprednisolone is a potent immune suppressor, and observations of anti-inflammatory effects potentially alleviating edema and the secondary injury cascade led to its use in the acute phase of spinal cord injury. Despite five RCTs with negative results in the primary outcome (45–47) and a significant increase in adverse events, there is still debate about its use because of beneficial effects seen in a subgroup of severe injuries receiving the treatment within eight hours after injury (47,118,140). Ganglioside, a cell membrane component, was initially successful in animal experiments and phase II trials but failed to show efficacy in phase III (141). Nimodipine, a calcium channel blocker, also failed in RCT (142), and so did gacyclidine (143).

GLIAL SCAR


Wallerian degeneration
Failed regeneration
Scar forming astrocytes
NG2-glia
Fibroblasts Pericytes
Cystic cavity
Cystic cavity

A number of potential candidates are currently in the translational process. Magnesium has shown preclinical efficacy (144), but a phase I/IIa trial was recently terminated by the sponsor due to insufficient enrollment (NCT01750684). SUN13837, a mimetic of basic fibroblast growth factor, recently completed phase I/IIa showing safety but not efficacy (145).

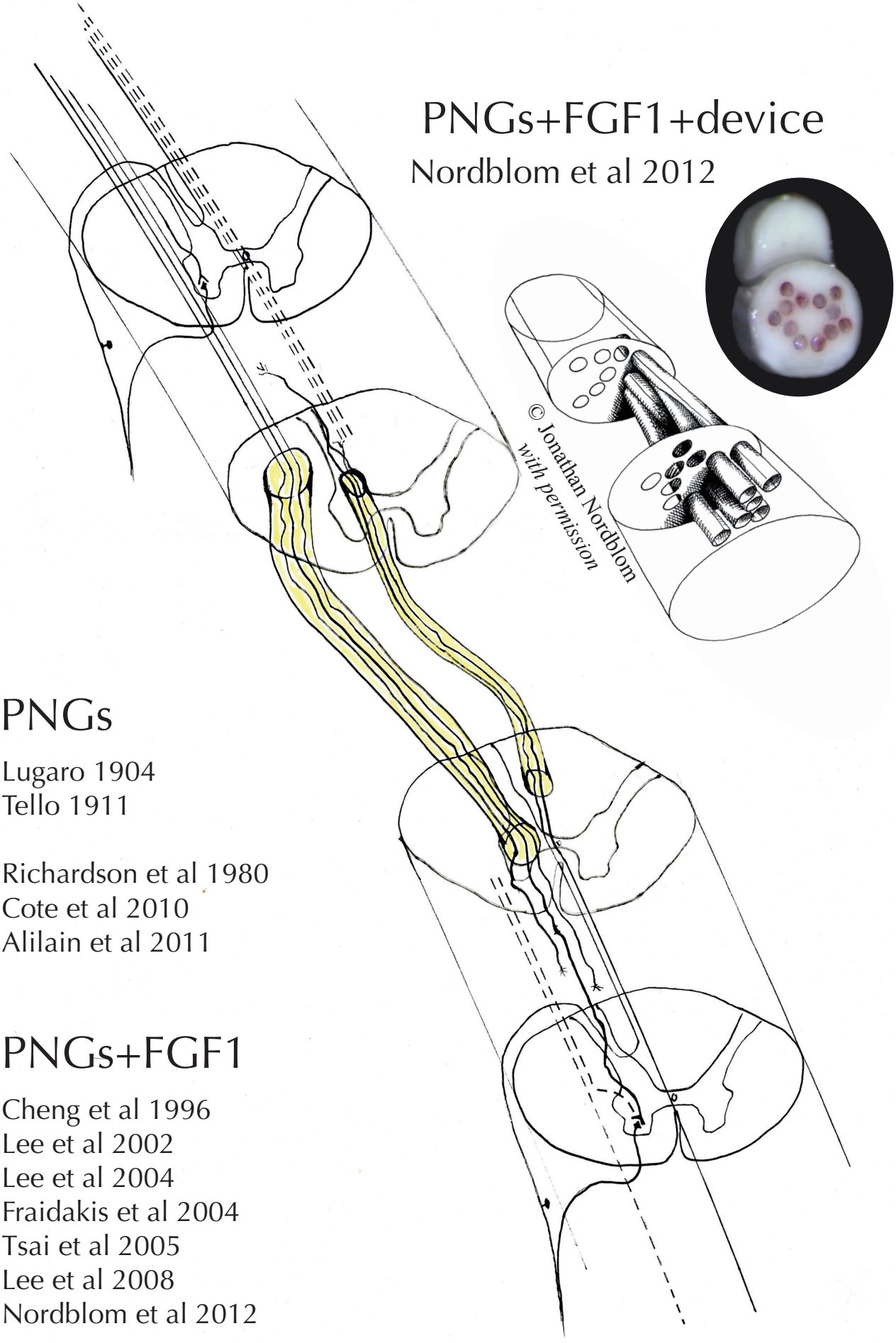
Anti-NOGO antibody has been tested and shown efficacy in many preclinical models of SCI (146–148) and was recently shown to be safe in phase I/IIa (149). Minocycline, an antibiotic, has been shown to be safe, and a trend towards positive outcome in cervical SCI was demonstrated in phase II (150). A phase III trial is recruiting patients but not completed (NCT01828203). Riluzole is a glutamate blocker currently registered for amyotrophic lateral sclerosis that has shown efficacy in animal models of SCI (151) and was proven to be safe in a phase I study (152). A phase II/III study is currently recruiting (NCT01597518). Cethrin/VX-210 has promoted axonal outgrowth after injury through blocking axonal growth inhibition via the Rho-ROCK pathway (153). A phase I study showed promising results when applied in fibrin sealant during decompressive surgery in the acute phase (154); an IIb/III study has been completed, but results have not been published (NCT02669849). Implantation of a bioresorbable polymer scaffold with known preclinical efficacy has been performed in a patient during acute decompression surgery (155); a clinical trial is ongoing, and 19 patients has been enrolled (NCT02138110).

Injection of stem cells for the treatment of spinal cord injury has shown preclinical potential across a wide variety of injuries, time points, and species (156–158). The approach has been proposed and tested for several decades, but has not resulted in approved interventions to date for SCI, despite clinical trials (159,160). Because positive effects have been seen after stem cell injections in the acute phase after experimental SCI despite limited number of cells surviving in the long term, it has been speculated that for some applications, the positive effect of stem cells could be purified and similar results obtained without the injection of cells. For other motives such as cell replacement of motor neurons lost in a cervical SCI, a stem cell graft is an attractive concept despite its many challenges (156).

WHEN FUNCTION IS LOST AND THE GLIAL SCAR ESTABLISHED

Scarring in many other parts of the body is functional, and the scarred tissue can acquire function to near pre-injury conditions. In humans and rats, a spinal cord injury commonly results in a post-traumatic fluid-filled cyst (161). The glial scar is found in the interface between cyst and normal spinal cord; it is thin (162) and, contrary to earlier belief, even softer than normal CNS tissue (163). The glial scar is important for reestablishing the blood-brain barrier and preventing further damage to tissue, but the resultant molecular environment of the glial scar makes regeneration of neurological function impossible (126,127). Regeneration attempts by injured axons fail, resulting in characteristic dystrophic end bulbs described by Cajal in the beginning of 20th century (164). These are commonly in direct contact with NG-2-positive cells via CSPG receptors (126).

CNS AXONS IN PNGs



PNGs+FGF1+device

Nordblom et al 2012

PNGs

Lugaro 1904
Tello 1911

Richardson et al 1980
Cote et al 2010
Alilain et al 2011

PNGs+FGF1

Cheng et al 1996
Lee et al 2002
Lee et al 2004
Fraidakis et al 2004
Tsai et al 2005
Lee et al 2008
Nordblom et al 2012

CNS AXONS CAN BYPASS THE GLIAL SCAR IN PERIPHERAL NERVE GRAFTS

The failed attempts of CNS axons to regenerate spontaneously through the glial scar after injury were known more than a century ago and described in detail by Cajal and others (164,165). Researchers at the time also recognized that severed axons in the peripheral nervous system had an intrinsic capacity for regenerating and re-establishing lost function, and that injured axons in the CNS would regenerate into peripheral nerve grafts positioned in the lesioned CNS. For an inspiring historical background, see the thesis by Fraidakis (166).

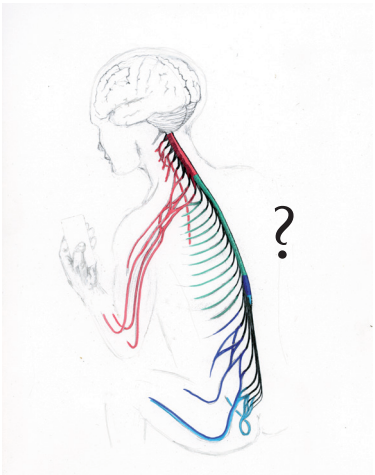
In the 1980s, the concept of CNS regeneration through peripheral nerve grafts was rediscovered and further characterized (167–169). In 1996 Cheng and colleagues further refined the experimental protocols by routing regenerating axons from white matter to grey matter on the opposite side of a complete thoracic spinal cord lesion by using autologous nerve grafts and adding fibroblast growth factor 1 (FGF1) in fibrin glue to increase neuronal sprouting (170). With this method, adult rats subjected to complete thoracic spinal cord transections regained some hindlimb function with time. Electrophysiological evidence and histological evidence of re-establishment of connections across the complete SCI were also present. The method has since shown similar results when performed in chronic complete injury (171), which is in line with the observation that a chronically injured axon will begin sprouting again after a re-axotomy of the dystropic end bulb after regeneration failure (172). The bridging strategy has also been repeated in several other labs (173–175), and similar methods of bridging with peripheral nerves in the spinal cord have been performed in a variety of experimental (176–178) and clinical (179–182) settings. Unfortunately, none of the clinical series reported in the literature has been designed as randomized controlled trials, and the level of evidence is therefore low.

A limitation for clinical translation of the pioneering work by Cheng et al. (1996) is the requirement of meticulous placement of the peripheral nerve grafts on the spinal cord transection's surfaces. Therefore, Nordblom et al. further developed the method (183) and finally applied a biodegradable guiding device made from calcium sulphate (184). The guiding device could be soaked in FGF1 and showed slow release of the growth factor during degradation (185). In rats subjected to complete thoracic spinal cord resection, the device-guided precision grafting resulted in robust return of electrophysiological response in hindlimbs, histological evidence of axonal regeneration, and some regain of hindlimb function (184). The minimal required dose of FGF1 in rats was also tested, showing that 7 ng was needed for return of motor-evoked potentials at four weeks after injury (186).

Encouraged by preclinical success in re-establishing connection across a complete thoracic spinal cord injury with autologous peripheral nerve grafts in a biodegradable guiding device, we set out to translate the preclinical method described by Cheng et al. and refined by Nordblom et al. to a clinical trial in SCI patients.

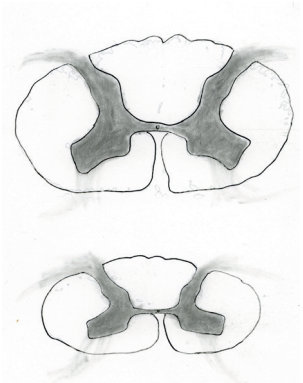
KNOWLEDGE GAP

I



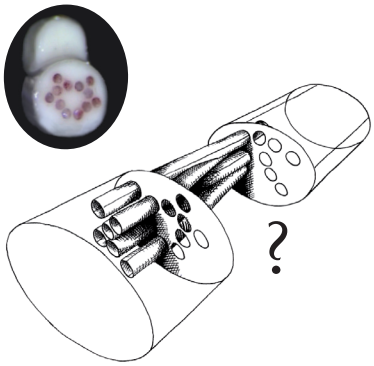
No precise motor scoring
in thoracic SCI

II



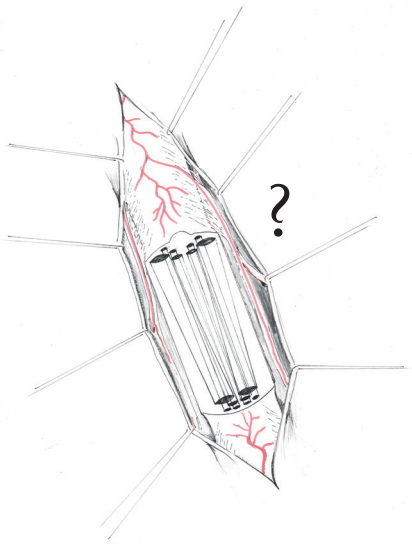
Knowledge on human
spinal cord size is scattered

III



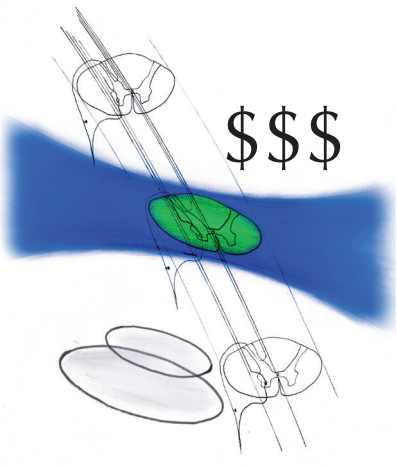
Preclinical guiding device
not suitable for human anatomy

IV



No previous clinical trial evaluating
glial scar resection and implantation

V



Light-sheet microscopy
is promising but expensive

OBSTACLES IN CLINICAL TRANSLATION OF A REGENERATION STRATEGY

In clinical trials, the safety of research subjects is the most important concern. Because of the anatomy of the spinal cord and the location of eloquent function, patients with complete (AIS-A) chronic spinal cord injury in the thoracic spinal cord are referred to as the "preferred patient group for clinical trials" (187,188). In the thoracic spinal cord, an unexpected adverse effect of the study intervention is the least likely to cause harm or deteriorate neurological function. This produces an important contradiction because no segmentally isolated key muscle function can be produced in approximately segments T2-L1 with clinical examination, and therefore determining the precise motor level is more or less impossible. This means that monitoring motor function in a clinical trial involving subjects with complete thoracic SCI is not possible with clinical examination alone. Additionally, knowledge of the *caudal* demarcation of the spinal cord injury is vital when applying a method that relies on re-establishment of connections across an injury gap. Anatomical evidence can be gathered with MRI, but a functional indicator of the extent of the injury would be required for reliable estimation of the length of the injury.

Further, applying a pre-fabricated medical device during surgery aiming to fit the cross-sectional surface of the spinal cord requires that the exact dimensions of the spinal cord is known in advance, either by non-invasive measurement (e.g. with MRI) in a patient or by producing a set of devices in different sizes covering the normal variability. Because the resolution of spinal cord MRI is currently insufficient when spinal instrumentation is present because of metal artefacts, the only remaining option is exact knowledge of spinal cord cross-sectional dimensions and variability. The literature contains several reports on spinal cord morphometry, but unfortunately different studies use diverse reference points for measurements, and the reported sizes of spinal cord varies considerably between studies.

To redesign the preclinical guiding device for a clinical trial, knowledge of cross-sectional size and variability of the human spinal cord would have to be combined with data on spinal tracts in humans for a resulting final design and sizing of a guiding device adapted for human SCI while retaining the key concepts from successful preclinical studies.

Reaching clinical trial in a translational process is a significant and collaborative undertaking requiring co-operation of multiple institutions, professions, and funding sources; rigorous external control of data quality; and safety of study subjects. The first and foremost concern is the safety of research subjects, and therefore thorough and early reporting of adverse events is of importance. Additionally, in a clinical trial evaluating a biodegradable medical device, confirming degradation of the device in the study subjects is pivotal.

Also, for the advancement of microscopic knowledge of spinal cord injury biology, lowering the cost of establishing a light-sheet microscope could make the technique more accessible for the research community.

AIMS

The overall aim of this thesis was to aid the preparation for clinical translation of a regeneration strategy for chronic and complete thoracic spinal cord injury by means of glial scar resection and autologous nerve graft transplantation using a biodegradable scaffold soaked in fibroblast growth factor 1.

Specifically, we sought to:

1) Develop a clinical neurophysiological method for precise investigation of segmental motor function in the thoracic spinal cord (paper I)

2) Define continuous estimates of human spinal cord segmental cross-sectional diameters and standard deviations from the literature (paper II)

3) Design a biodegradable guiding scaffold for human thoracic spinal cord dimensions and define a set of sizes covering the variability in size encountered in humans (paper III)

4) Describe the early adverse events in the ongoing clinical trial "Safety and Efficacy of SCo806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects" and the biodegradation of the guiding scaffold as observed by computerized tomography (paper IV)

For more precise investigation of spinal cord pathology and potential future attempts to expand spinal cord regeneration to for example incomplete or cervical injuries, we also endeavored to:

5) Construct a cost-effective light-sheet microscope by modification of an outdated microarray scanner for whole-mount imaging of central nervous system tissue (paper V)

MATERIALS AND METHODS

FORMAL APPROVAL OF THE RESEARCH

Papers I and IV involve human subjects. Both studies were approved by the Independent Ethics Committee in Stockholm (Etikprövningsnämnden) under permit 2010/344-31/2 (paper I) and permit 2013/2257-31/1 and 2015/1436-31 (paper IV). The study reported in paper IV was also approved by the Swedish Medical Products Agency Study Protocol SC0806-A101, version 10

EudraCT number 2013-000906-52, and registered at ClinicalTrials.gov under identifier NCT02490501 before the inclusion of study subjects. The study detailed in paper IV is ongoing, and only short-term safety and degradation of the guiding device is reported in paper IV.

Studies I and IV were performed at Karolinska University Hospital, Solna in the departments of Neurosurgery, Neurology, and Neurophysiology. Initial screening, follow-up, and rehabilitation in study IV was performed at the Spinalis Foundation, Solna. Radiology was performed at the Karolinska University Hospital, Solna and Radiology Center, Medicinsk Röntgen Hötorget, Stockholm. All subjects in papers I and IV signed written informed consent forms prior to participating in the studies.

Paper V involved rats as experimental animals. The study was approved by the regional ethics committee in Stockholm (Stockholms djurförsöketiska nämnd) under permit numbers N104/15 and 14274-2017. All experiments were performed at animal facilities at Karolinska University Hospital, Solna.

Papers II and III did not undergo formal ethical approval because they only involved data from already published sources. The ethics of papers II and III rely in part on the ethics of the published material used as data sources, which we have found no reason to question.

HUMAN RESEARCH SUBJECTS (PAPERS I-IV)

Paper I included five male subjects with complete chronic thoracic spinal cord injury (AIS-A) at levels T3-T7 recruited through the spinal cord injury ward at Karolinska University Hospital, Solna. Eligibility was based on medical records and clinical neurological examination.

Paper II included data from 631 study subjects without spinal cord pathology gathered from 10 published studies on spinal cord cross-sectional size (189–198), two studies on spinal cord neuronal segment length (197,199), three studies on human vertebral segment length (200–202), and two studies relating measures of the spinal cord and vertebrae (203,204).

Paper III used the summary data calculated in paper II and added data from two published series of histological sections of two human spinal cords without spinal cord pathology (194,205).

Paper IV describes early adverse events reported from the ongoing clinical trial "Safety and Efficacy of SC0806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects." As of November 2018, nine subjects with complete chronic thoracic spinal cord injury (AIS-A) at levels T4-T11 have been included. Six were randomized to glial scar resection, implantation, and rehabilitation, and three were randomized to rehabilitation only. Study subjects were recruited through rehabilitation centers in Sweden. After initial screening and study information, subjects who give informed written consent and are provisionally eligible are further screened for eligibility at Karolinska University Hospital.

META-ANALYSIS AND DESIGN OF GUIDING DEVICE (PAPERS II AND III)

Studies and data

We searched PubMed for original research publications reporting morphometric data on the human spinal cord. Studies not found in PubMed but referred to in the included studies were also added. We included three studies presenting the length of the vertebral bony segments in table 1b (200–202).

To define the white-to-grey matter delineation in the thoracic spinal cord, we used two representative series of microscopic slides covering the entire human spinal cord segmentally found in the literature (194,205). The location of spinal tracts was estimated based on schematic representations found in the literature in combination with degeneration studies.

Software and extraction of data from published studies

Data were gathered in Microsoft Excel and stored as comma-separated values (.csv), all calculations were performed in R (206), and graphs were produced with the ggplot2 and cowplot packages (207,208). Bootstrapping was performed with the boot package (209).

Most of the studies included did not present the raw data from their measurements; instead, averages and standard deviations were provided. Some of the studies did not present their data in numerical format but only in a graphical format. To ensure correct extraction of data from these studies, we imported images of the graphs into a CAD program (Rhino 5 for Mac, Robert McNeel & Associates) and used the internal measurements tool to extract the exact values from the graphs. All raw data, results from simulation, and codes are available upon request.

Relative lengths of spinal cord neuronal and vertebral bony segments

Using the data from the studies in table 1b, we calculated the relative length of each spinal cord neuronal segment by simply dividing the length of each segment by the total length of the spinal cord. When estimating the segmental diameter, the measurements from the different studies were weighted according to the number of subjects (i.e. individuals) in the respective study.

Using the data from the studies in table 1b, we also calculated the relative length of each vertebral bony segment using the same method as for the neuronal segments described above. A vertebral bony segment was defined as the vertebrae and half of the two adjacent intervertebral discs. The discs were assumed to increase in size proportionally to the vertebrae.

There were no measurements for vertebral segments C1 and C2 in the studies that we found. Their respective ratios were approximated by aligning vertebral bony segments with spinal cord neuronal segments in the cervical region according to Cadotte and colleagues (203). Specifically, the distance between the midpoint of spinal cord neuronal segment C3 and vertebral bony segment C3 was set to 1.3 times the distance between spinal cord neuronal segments C3 and C4. Finally, we assumed that both the spinal cord and the vertebral column terminated at the same cranial level and divided the distance equally between C1 and C2 vertebral bony segments. Therefore, our calculated relative size of C1 and C2 should be considered approximations and interpreted with care.

Relative positioning of spinal cord neuronal and vertebral bony segments

To align the spinal cord neuronal segments with the vertebral bony segments, we multiplied all cumulative percentages for vertebral bony segments by 1.29. This scaling factor was calculated by dividing the cumulative percentage of the entire spinal cord (100%) with the cumulative percentage of the vertebral column at vertebral bony segment L1. This new scaling of vertebral bony segments set the caudal end of the L1 vertebral bony segment equal to the caudal end of spinal cord neuronal segment S5. The positioning depends on the knowledge of the relative positions of the C3 and C4

spinal cord neuronal segments to the C3 vertebral bony segment presented in the study by Cadotte and colleagues (203) and the level of termination of the spinal cord between vertebral bony segments L1 and L2 (204).

The relative positions of the segments were used to find the correct relative positions of each cross-sectional measurement along a normalized craniocaudal axis of the human spinal cord. Each measurement was placed as closely to the anatomical position described by the original authors as possible with respect to the type of segmental reference used in the study (spinal cord neuronal segment or vertebral bony segment), as well as the positioning on that specific segment (cranial end of segment, midpoint of segment, or caudal end of segment).

To estimate the effect of adjusting the craniocaudal position of measurements of the transverse diameter of the human cervical spinal cord, we fitted a linear regression model before and after correction of craniocaudal position:

$$Transverse\ diameter \sim \beta_1 * position + \beta_2 * position^2 + \beta_3 * study$$

The squared term was added because the cervical spinal cord transverse diameter approximates the shape of a second-degree polynomial, and the dummy term *study* was added to correct for differences in intercept between the studies. Adjusted R-squared was used as a measure of alignment of the cervical intumescences between studies. Confidence intervals for adjusted R-squared were estimated using a 1000-iteration bootstrap.

Weighted averages of spinal cord cross-sectional diameters and variance

To combine the cross-sectional measurements of the human spinal cord from all studies into single estimates, we calculated a moving weighted average. First, measurements from all studies were aligned along their position on our corrected craniocaudal axis described above. Thereafter, starting at the cranial end, four consecutive measurements of spinal cord diameter were combined into a single average, weighted by the number of subjects in the underlying studies for the four included measurements. The average position along the craniocaudal axis of the four measurements was used as the new position for the weighted average. Next, the most cranial of the four measurements was dropped, and the closest measurement caudal to the three remaining measurements was included to create a new group of four measurements, with a new weighted average and a new position along the craniocaudal axis.

Moving weighted variances were calculated using the same method as described for the moving weighted averages. The calculated variances were then converted to weighted standard deviations.

Continuous estimates of spinal cord cross-sectional diameters and variance

To construct continuous population estimates and achieve further smoothing, a generalized additive model was used to fit the weighted averages and weighted standard deviations. We used the smoothing function of ggplot2 (208) in R (206) with the formula y ~ s(x, k = 12), allowing for a 12-degree polynomial function to fit the data.

To facilitate comparison between our continuous population estimates and other studies, we extracted values for each spinal cord neuronal segment as well as each vertebral bony segment. The number of subjects measured for a given segment was defined as the total number of subjects included in any study with a calculated craniocaudal position inside the cranial and caudal limits of the segment in question. This was used as an approximation of sample size, as there is no obvious way of calculating exact sample size for different portions of a smoothing function.

Simulation of a population of spinal cord sizes

To facilitate visualizations and calculations, the continuous estimates from paper II were used to generate a theoretical sample of spinal cords with the same mean and distribution as in the published paper. Two hundred samples per spinal cord level were generated using the mvrnorm()-function in R with the package "MASS" (206,210). The correlation of the bivariate Gaussian distributions generated was set differently for each segment in a continuous fashion, dependent on transverse diameter. These ranged from high correlation (0.9) in the small segments (sacral spinal cord) to lower correlation (0.4) in the large segments (cervical cord). This was done to satisfy the shape constraint observed in the raw data of spinal cord sizes (i.e. the spinal cord can be neither too "flat" nor too "round") (197).

Elliptical interfaces for guiding devices covering the thoracic spinal cord

Given the continuous estimate of spinal cord segments T2 to T12, we chose seven elliptical shapes that covered these thoracic spinal cord segments by using three different ellipses with a ratio between anteroposterior and transverse diameter (RAPT) equal to the mean RAPT of the thoracic spinal cord (termed "normal" shape), an additional two ellipses with a rounder shape than the mean RAPT (termed "round" shape), and two ellipses with a flatter shape than the mean RAPT (termed "flat" shape). The ellipses were spaced symmetrically and placed to cover the central part of the population estimates.

Another three large ellipses were added—one for each shape—to prepare for the event of spinal cord swelling due to surgical manipulation. These extra ellipses were not included in the error-of-fit calculations since they were intended as an extra safety measure.

Error-of-fit between thoracic spinal cord segments and guiding devices

To calculate the error of fit between our set of guiding device sizes and the simulated spinal cord sizes, we assigned each simulated spinal cord segment (between T2 and T12) to the best-fitting guiding device in our set of seven sizes. The best-fitting guiding device was chosen by attempting to minimize both transverse and anteroposterior difference between the simulated segment and the elliptical shape of the guiding device. This was accomplished by minimizing the square root of the sum of the squares of the transverse and anteroposterior error (equal to minimizing the Euclidian distance between the simulated spinal cord segment and the guiding device in a two-dimensional space representing transverse and anteroposterior diameter). After the best-fitting guiding device was chosen for every simulated spinal cord size, we calculated the transverse and anteroposterior error separately, as well as the mismatch in area. This was done for every simulated spinal cord segment, which resulted in 200 segments per level between T2 and T12 for a total of 2,200 simulated spinal cord sizes.

Creating a vector model of the human spinal cord

Images covering the entire human spinal cord were available in a digital format and were imported in a CAD program (Rhinoceros for Mac, version 5). The outline of the spinal cord and the delineation between white and grey matter were traced manually to create a vector model. The midline of the spinal cord was identified, and both traced halves were averaged to yield symmetric white-to-grey matter delineations for each segmental level of each spinal cord series. Furthermore, each segmental image was scaled to the average transverse and anteroposterior diameter for that segment using the data collected from paper II. Thereafter, segments T2 to T12 were averaged in both spinal cord series, and both thoracic averages were combined into a final average representation of the delineation between white and grey matter in the human thoracic spinal cord.

Designing the guiding device interfaces and channels

The device was designed in a CAD program (Rhinoceros for Mac, version 5). The guiding channels of the device were chosen using the vector model of the spinal cord and anatomical knowledge of the spinal cord tracts in humans. Channels were designed to start at a relevant descending (e.g. corticospinal tract) or ascending (e.g. dorsal column) tract and to run obliquely through the device to reach grey matter at the other side of the device. Channels were spaced so as not to intersect each other and to leave a wall of material of at least 0.4 mm between channels.

Alignment of guiding device channels and spinal cord white matter tracts

To assess the alignment of the device channels, the vector model of the average thoracic spinal cord and the guiding device interfaces were imported in R. The spinal cord shape was then scaled to the dimensions of each simulated thoracic spinal cord segment and overlaid on the shape of the device that showed the best fit for the particular simulated spinal cord segment. The results were assessed qualitatively.

Determining the final set of guiding devices

From the design process described above, we choose seven guiding device sizes and an extra three larger sizes for a total of 10 interface sizes. Because the spinal cord is mobile to some extent in the spinal canal, the length of the guiding device was chosen with 5 mm increments from 15 mm to 40 mm, covering the shorter range of thoracic spinal cord injuries (211). This resulted in a total of 60 guiding devices (10 interfaces x 6 lengths).

METHODS USED IN THE CLINICAL TRIAL (PAPERS I AND IV)

Neurological examination

Neurological examination was performed on all study subjects in papers I and IV by a senior consultant neurologist with experience in spinal cord injuries. The examination was performed according to the AIS classification.

Neurophysiology

The neurophysiological method used in paper I was also employed as part of the preoperative workup and inclusion criteria for paper IV. All examinations in both studies were performed by the same senior consultant neurophysiologist, blinded to the results from the clinical examination as well as imaging such as MRI and fMRI.

In brief, bipolar needle electromyography (EMG) recordings were obtained from intercostal muscles near the neurological level of injury on both sides of the midline. Two clinicians independently counted and marked intercostal spaces along the sternum and laterally along the costal margin. In paper I, an indicator was placed during MRI examination to validate the true intercostal space. This was not performed in study IV because after testing in study I, manual counting was found to be reliable. The EMG recordings were obtained in rest, during voluntary activation through head lift and spastic activation of lower limbs.

To avoid the influence of the pectoralis major and serratus anterior muscles, needle placement was selected along the edge of the sternum and along the costal margin. We also let the subjects specifically activate the pectoralis major and serratus anterior muscles to evaluate their signal in each needle position.

As part of the screening procedure in study IV, to investigate any residual subclinical function, we evaluated motor-evoked potentials in the lower limbs elicited by transcranial magnetic stimulation of the motor cortex. Sensory-evoked potentials were also investigated by electrical stimulation of the lower limbs and registration with surface electrodes on the scalp overlying the sensory cortex.

During surgery in paper IV, monopolar recording needles were placed in each intercostal space approximately two segments above and below the neurological level of injury, with reference electrodes in the same intercostal space approximately two centimeters laterally. Using these electrodes, transcranial MEP was recorded in the intercostal spaces, in addition to direct stimulations on the ventral roots during surgery as well as dorsal roots to elicit an H-reflex.

Clinical imaging

In papers I and IV, magnetic resonance imaging (MRI) was performed on all study subjects to investigate the anatomy of the spinal cord injury area. Since all included subjects had been operated on with spinal fixation at the time of their spinal cord injury, normal MRI sequences were unusable in the injury area because of metal artifacts. During the data collection of paper I, we found a protocol that was less prone to metal artifacts. The imaging was performed on a 1.5-tesla whole-body MRI scanner (Philips Intera Master, Best, The Netherlands) with a 15 ch spine coil (Medical Advances, Milwaukee, WI, USA). The diagnostic imaging protocol was acquired in a sagittal plane using a customized 3D TSE T2W isotropic voxel pulse sequence. The voxel size was 1.0x1.0x1.0 mm with the following parameters: repetition time 2000 ms, echo time 120 ms, flip angle 90 degrees, and field of view 300 mm.

In study IV, functional MRI during sensory stimulation of the lower limbs is performed during screening to investigate potential residual subclinical function. Imaging was performed on a clinical 3 Tesla Discovery 750w with an 8HRBRAIN-coil (GE Healthcare, Waukesha, USA). Images of the

cortex were acquired in a 2D coronal sequence with the following parameters: 41 slices in 128 time points, field of view 240x240 mm, slice thickness 3.6 mm, repetition time 2500 ms, echo time 30 ms, and flip angle 90 degrees.

In paper IV, subjects operated on in Part A of the clinical trial underwent postoperative CT scans at 1, 14, and 60 days after surgery in the region of the SCI device to study possible adverse events and the degree of resorption of the device. Images were acquired on Discovery CT750 (GE Healthcare, Waukesha, USA) at 120 kV in helical mode and reconstructed with slice thickness 0.625 mm, slice spacing 0.325 mm, field of view 150x150 mm, and 512x512 voxels per slice.

Interpretation of clinical imaging

The length of the discontinuity of the spinal cord was determined by cranio-caudal examination of the spinal cord in the transverse plane. The cranial marker was set where signs of neural tissue and exiting spinal nerves disappeared, and the caudal marker was set where these signs reappeared.

In paper IV, the CT scans from different time-points postoperatively were co-registered for each study subject with three degrees of freedom (XYZ) using anatomical landmarks in FIJI (212). Thereafter, a cylindrical region of interest (ROI) was placed around the device in the first post-operative image (one day after surgery), and the intensity values of each voxel in the ROI were exported to a .csv file. The same ROI was applied to all co-registered images from the same subject, and the voxels for each time-point were exported. The raw voxel data were plotted as histograms of the different time-points.

Data Monitoring Committee

The study is monitored by an independent Data-Monitoring Committee (DMC) with continuous access to Serious Adverse Events (SAEs) and further information at their request. The Data-Monitoring Committee consists of two physicians (one neurosurgeon and one neurologist) and a statistician. The DMC is independent from the clinical trial and the sponsor of the clinical trial, BioArctic AB, except in their role as members of the DMC. The DMC is also tasked with the duty of performing interim analyses at three pre-defined points during the clinical trial and evaluating safety and tolerability as stated in the Clinical Study Protocol (CSP). The members of the DMC also function as the eligibility committee as described below.

Eligibility committee and final inclusion

If screening confirms a potential subject to be included in the clinical trial, all screening information is sent de-identified to the eligibility committee for independent review. The eligibility committee then gives a recommendation whether to include the patient. After screening, subjects are once again informed of potential adverse effects of the study procedure and offered participation in the clinical trial.

Key inclusion criteria are:

1. Traumatic Spinal Cord Injury.
2. Male or female subjects aged between 18 and 65 years.
3. Complete SCI (ASIA Impairment Scale level A, no voluntary bladder function, negative motor and sensory evoked potentials).
4. A single spinal cord lesion injury at the neurologic level between T2-T11.
5. A Baseline MRI that indicates a pathology consistent with a traumatic SCI
6. Minimum of 4 months and maximum 10 years post injury with no evidence of neurological improvement prior to implantation surgery unless there is a complete anatomical cut-off of the spinal cord.

Key exclusion criteria are:

1. Other life-threatening injury.
2. Serious co-existing medical condition or mental disorder.
3. Results from neurophysiological examination preoperatively are inconsistent with a spinal cord injury of one thoracic segment or less.

Study design and randomization

After inclusion in the study, subjects are randomized to receive either surgery and rehabilitation or rehabilitation only. The ongoing study is designed to include a total of 27 subjects in three parts. A weighted block-randomization protocol is used, resulting in six subjects randomized to surgery and rehabilitation and three subjects randomized to rehabilitation only for each part. Subjects randomized to surgery are operated on in a staggered fashion, allowing at least one month between surgeries to identify potential adverse events in the follow-up period before making the decision to operate on the next subject.

According to protocol, subjects randomized to rehabilitation only will be offered surgery and rehabilitation after completion of the first rehabilitation period of 18 months, provided that efficacy of the treatment has been shown in the study.

Surgery

The surgeries of the six subjects operated on in Part A of the ongoing clinical trial presented in paper IV were performed at the department of Neurosurgery, Karolinska University Hospital, Solna. Subjects are admitted the day before surgery, and the immediate preoperative workup includes vital stats, blood samples, pregnancy test (in the case of a female subject of child-bearing age), and anti-FGF1 antibodies. After all relevant clinical information is gathered, the final decision to perform surgery is made by the principal investigator.

In the ongoing trial, after general anesthesia, subjects are placed in the prone position. The injured thoracic spinal cord is exposed, and the exact demarcation of the injury is determined through visual inspection in the operating microscope together with electrical stimulation of the exposed spinal nerves and measurements of EMG in intercostal muscles. The ventral roots are stimulated to verify the spinal cord segmental level and for final evaluation of the secondary motor neuron pool in the ventral horn, the dorsal roots are stimulated, and the intercostal EMG is monitored for reflex activation. An intact reflex upon dorsal root stimulation is considered indicative of a functioning spinal cord segment. If the injury is confirmed to be suitable for implantation (i.e. in accordance with the preoperative workup and of suitable length), the injured spinal cord is transected, and the glial scar is removed. The exact dimensions of the transected surfaces of the spinal cord are measured with custom-made instrumentation to find the best-fitting device from a set of 60 devices (10 different sizes and shapes and six different lengths, paper III). The device chosen for implantation is soaked in human recombinant fibroblast growth factor 1 for 60 minutes. During soaking, one of the subject's sural nerves is dissected, and a portion of it is removed. The nerve is separated in fascicles suitable to match the dimensions of the different channels of the device; when soaking is finished, the fascicles are placed in the guiding channels of the device. After loading with autologous peripheral nerve grafts, the guiding device is placed in the resection gap between the two transected ends of the spinal cord and secured with sutures to the anterior aspect of the dura. A duraplasty is then performed to allow optimal circulation of cerebrospinal fluid around the device and spinal cord, and the wound is closed in anatomical layers. To ensure required air cleanliness (ultraclean air) and decrease the risk of contamination by airborne bacteria, mobile laminar airflow units (Toul Meditech, Sweden) are used to protect instruments and wound areas throughout the procedure (213).

After surgery, subjects are transferred to the neurointensive care unit and, when stabilized, transferred to a standard neurosurgical ward. Length of hospitalization is individualized according to the medical needs of the subject. At discharge, subjects are transferred to the rehabilitation center.

Rehabilitation and follow-up

All included subjects, regardless of randomization to surgery and rehabilitation or rehabilitation only, receive specific robotic-assisted body weight-supported treadmill training in the Lokomat® system (56) three times a week for 18 months and an optional extension of 12 months of training. All follow-up visits are carried out at the rehabilitation center and include regular safety monitoring at planned appointments with a study physician. A follow-up visit 12 months after study completion has been added for all patients in the active arm.

Reporting of Adverse Events and Serious Adverse Events

Reporting and registration of adverse events (AEs) started the day of signing the informed consent for participating in the clinical trial. Any untoward medical occurrence, unintended disease or injury, or untoward sign (including an abnormal laboratory finding) in subjects is considered an AE, regardless of relation to the study intervention. An AE is considered a serious adverse event (SAE) if it fulfills any of the following:

- results in death
- results in a life-threatening illness
- requires in-patient hospitalization or prolongs an existing hospitalization. To fulfill this criterion, the patient is to be admitted to in-patient care for medical reasons
- results in persistent or significant disability/incapacity or impairment of body structures
- is a congenital anomaly/birth defect (in the child of a subject who is participating in the study
- results in medical or surgical intervention to prevent life-threatening illness or injury
- results in fever (>39ºC) during the first postoperative month
- is another important medical event or significant medical condition that may not fulfill the above seriousness criteria. This includes also medically significant laboratory abnormalities with or without symptoms

In this preliminary report from an ongoing clinical trial, all data will be reported up to 60 days after surgery or inclusion with the exception of SAEs, for which all available data will be reported (i.e. any subject experiencing an SAE after the 60-day period detailed in this report will be also be reported as an SAE).

CONSTRUCTION OF A MICROSCOPE FROM A MICROARRAY SCANNER

Experimental animals

Animals were kept in enriched cages with free access to water and food in a room with a 12:12h light cycle. All experimental procedures were approved by the local ethics committee for animal experiments in Stockholm (ethical permit number 14274-2017 and N104/14).

Hypoglossal nerve injury was induced in one adult Sprague-Dawley rat (body weight 210 g). The rat was anesthetized using medetomidin 0.5 mg/kg (Domitor ® Vet., Orion Pharma Animal Health, 1 mg/ml) and ketamine 75 mg/kg (Ketador vet., Salfarm Scandinavia, 100 mg/ml). Sodium chloride was administrated s.c. preoperatively and postoperatively to compensate for dehydration due to surgery. Using a hair trimmer (Aesculap, ISIS 273278), the fur covering the front of the neck was removed. The skin and muscles were incised and separated using a scalpel to expose the hypoglossal nerve on the right side. The nerve was avulsed using small forceps, and the skin was closed using running sutures (Vicryl, 4.0).

Seven days following injury, the rat was administrated a lethal dose of pentobarbitalnatrium (300 mg/kg). Using a peristaltic pump (Watson Marlow, 120S), the rat was first perfused with normal saline (4°C) followed by 4% PFA. A second, uninjured rat was also sacrificed. The spinal cords and brainstems were carefully dissected and post-fixated in 4% PFA at 4°C overnight.

Tissue clearing and staining

The perfused and post-fixed tissue was sectioned on a vibratome (752M 152 Vibroslice, Campden Instruments, UK) in 0.5-1 mm thick slices. The tissue was optically cleared using a modified passive CLARITY protocol (11). Briefly, sections were placed in 4% acrylamide (A3553, Sigma Aldrich, Germany) overnight (12h). Tissue specimens were then degassed using a vacuum pump to evacuate the air in an air-tight chamber. Thereafter, the chamber was filled with nitrogen. When pressure in the chamber corresponded to the ambient pressure, the lid was opened, and the tubes were quickly closed. Polymerization was induced by incubation at 37°C for three hours. The sections were washed for a week in 8% sodium dodecyl sulfate (SDS) (L3771, Sigma Aldrich, Germany).

The section of the brainstem was stained for 48 hours with the primary antibody Iba1-rabbit. After three rinse cycles in PBS with 3% Triton X-100 (X100, Sigma Aldrich, Germany) for 15 min each, the specimen was incubated in the secondary antibody and the cell nuclei stain for propidium iodide for another 48 hours and then washed again in PBS.

For imaging, Refractive Index Matching Solution (RIMS) was prepared using 40 g of Histodenz (D2158, Sigma Aldrich, Germany) mixed in 30 ml of 0.02 M phosphate buffer (in-house) with 0.1% Tween-20 (P1379, Sigma Aldrich, Germany) and 0.01% sodium azide (S2002, Sigma Aldrich, Germany). The pH was adjusted to 7.5 with NaOH (S8045, Sigma Aldrich, Germany). This resulted in a final concentration of 88% w/v Histodenz. Tissue specimens were incubated for 12h in RIMS before imaging (214).

Parts and outline of the light-sheet fluorescence microscope

A light-sheet fluorescence microscope (LSFM) was constructed through modification of an HP Agilent micro-array scanner GeneArray G2500A. The optical path forming the focused laser beam originated from the scanner itself. In the scanner, all parts were installed on a metric optical breadboard (Model no 78-25555-01, Technical manufacturing Corporation, USA) measuring 38 x 48 cm. The 0.62 mm collimated beam from a 10 mW argon-ion laser (2211-10SLHP, JDS Uniphase, USA), wavelength 488 nm, was expanded using a 10x beam expander (Rodenstock, Germany), reflected on a galvo-scanner mirror (model 6860*231, Cambridge Technology, USA), and focused using a f=50 mm Theta lens (Rodenstock, Germany).

Controlling the laser and galvo-scanner

The argon-ion laser of the scanner could be started by shorting two wires of the control cable (completing the interlock-circuit, pins 1 and 3) and applying 5 volts of direct current to another cable (pin 2). Thereafter, laser intensity could be controlled by varying the resistance between a third pair of wires between 0 and 1000 ohms (pins 7 and 11).

Through a small modification of the scanner logic board by shorting a pin on one IC by clamping with a crocodile clip, the amplifier circuit for controlling the galvo-scanner could be controlled with an external function generator (model UTG9003A, UNI-T, China). The galvo-scanner was driven with a 200 Hz sinus wave: amplitude controlled the height of the light-sheet in the Y-axis, and voltage offset controlled the position of the light-sheet in the Y-axis.

The focused and scanned beam formed the light-sheet that passed through a 170 μm thick microscopic cover-glass into a custom-made chamber, equipped with a NA0.3, 10x water immersion objective (Olympus, Japan) and situated perpendicular to the excitation axis. The image projected at infinity from the detection objective was passed through a StopLine® quad-notch filter (Semrock, USA) and collected with a 200 mm f/4 lens (Nikon, Japan) mounted on an EOS 6D DSLR (Canon, Japan). The tissue was mounted between two cover glasses (Marienfeld, 010243), glued to a 1 mm thick glass slide (VWR, SuperFrost® Plus, 48311-703), and mounted at a 45-degree angle to the light-sheet and detection axis. Moving of the tissue was accomplished using a motorized micromanipulator (Model 5171, Eppendorf, USA). The joystick of the motorized micromanipulator was controlled with an attached manual micromanipulator (Model M33, World Precision Instruments, USA) with 0.1 mm resolution. This was done to enable precise control of the translation speed of the specimen during imaging by setting and maintaining an exact angle of the control joystick.

Focal spot size

To calculate the ideal $1/e^2$ width of the focused laser beam forming the light-sheet, we used the formula $2\omega = \left(\frac{4}{\pi}\right)\lambda\left(\frac{f}{d}\right)$, where λ is wavelength, f is focal length of the focusing lens, and d is beam diameter at the lens aperture. This was compared to actual measurements made in the microscope of the focused laser without scanning the focal spot in the Y-axis.

Imaging

The custom-made imaging chamber was loaded with freshly prepared RIMS (214). In order to reduce the occurrence of light-scattering bubbles, the chamber was filled ~30 min prior to imaging. To enable recording of 14-bit raw sensor data in video mode, the open-source add-on software Magic Lantern© was installed on the camera using a 64GB 1000x CompactFlash® (Komputerbay Professional). The continuously acquired images were temporarily stored on the same flash card during imaging and then converted to cinemaDNG-sequences using the software RawMagic 1.0. Resolution, sensitivity of the image sensor (ISO-number), and frame rate were chosen based on the specific imaging conditions.

Specimen Z-translation

To enable acquisition of isotropic voxels through an image stack, movement in the Z-axis during imaging must match the lateral resolution multiplied by the frame rate. Because we used a micromanipulator with a joystick interface, the relation between joystick angle and Z-axis speed had to be determined empirically. We used a 1D micromanipulator with 0.1 mm resolution attached to the joystick to set a specific joystick angle and recorded images of a Bürker Chamber Glass slide with a printed square pattern (Heinz-Herenz, Hamburg, Germany) translated along the Z-axis of the

microscope. We then calculated the mismatch in lateral and Z-axis resolution using FIJI (212) for a number of different joystick angles. The results obtained were fitted to a third-degree polynomial function using R (206), and an interactive interface was created using Shiny (215) to obtain the correct settings quickly during imaging (https://frostell.shinyapps.io/speedSetting).

Image processing

Raw sensor data acquired with Magic Lantern were converted to the CinemaDNG format using Raw Magic 1.0. Bayer interpolation was applied in DaVinci Resolve Lite (Blackmagic Design, USA). Images were exported from DaVinci as 16-bit TIFF images and imported into ImageJ. Using a custom-written macro, the images were converted (resliced) from being orthogonal to being longitudinal in relation to the tissue. In the final step, the images were shifted (translated) in order to realign the images and reconstruct the original shape of the tissue. Specifically, prior to translation, the images formed a representation of the tissue resembling a parallelogram. Translation restored the rectangular shape of the tissue by shifting the intermutual relation between the images. A Mac Pro with a 3 GHz 8-core Intel Xenon E5 processor was used for image processing. The workstation was equipped with 64 GB 1866 MHz DDR3 ECC memory and an AMD FirePro D500 3072 MB graphics cards.

Automatic cell counting

Cell nuclei overlapping the primary antibody staining (Iba1) were counted automatically using FIJI (212). Specifically, we first applied a region of interest around the hypoglossal nuclei, multiplied voxel values from the red channel (representing cell nuclei) by voxel values from the green channel (representing Iba1 positive cells), and then applied a threshold and counted the cells with the 3D simple segmentation tool in FIJI (212). Data on the XYZ position of each identified cell were exported as a .csv file and imported in R (206) for plotting. Data presentation was conducted using packages ggplot2, cowplot, and shiny (215).

Cost-estimation of the microscope

Because most of the parts used in the microscope were already in our lab when we started the construction, our estimation of the costs of the parts represents a best-guess based on what we considered an average price on common markets for used laboratory equipment (e.g. ebay.com).

RESULTS

The papers in this thesis describe parts of the translational process of a preclinical regeneration strategy for complete chronic thoracic spinal cord injury to a clinical trial (papers I-III), the short-term safety reported in the ongoing clinical trial "Safety and Efficacy of SCi806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects" (paper IV), as well as the construction of a low-cost light-sheet microscope (paper V).

PAPER I – INTERCOSTAL EMG PRECISELY DEMARCATES A THORACIC SCI

Five male patients with complete chronic SCI in the thoracic cord were included. Written and informed consent was acquired from all subjects. Age of patients were 32-50 years (median 35), 1-4 years post injury (median 3). Three patients were injured in motorcycle accidents, one patient in a motor vehicle accident and one in an accident with a hang-glider. All patients presented as AISA A and remained so throughout the clinical course. None of the patients had a clinical history of frequent autonomic dysreflexia.

Clinical examination

Patients underwent clinical examination to confirm that all inclusion criteria were met. They were all determined ASIA A as their clinical history suggested. Neurological level was T3-T6 (median T5) and lower body spasticity was present in all patients.

Neurophysiology

To assess motor function of the thoracic spinal cord bipolar needle EMG registrations in the intercostal muscles were performed. Three distinct patterns were recognized: Above the level of SCI normal, voluntarily activated motor unit potentials (MUPs) appeared . At, or close to, the level of sensory loss patients presented a varying number of intercostal spaces with spontaneous EMG activity with fibrillation potentials and positive sharp waves. Below the level of injury there were once again normal MUPs, generated in concert with spastic activation of lower limbs. The registrations and number of denervated segments from the five individual patients are presented in paper I.

The patients' sensation of the needle insertion was used to determine the sensory level. It was coherent with motor level in two patients, was found one segment above voluntary motor level in two patients, and one segment below motor level in one patient.

MRI

MRI was performed in all patients to give an anatomical overview of the individual injury and facilitate interpretation of results from neurophysiology. In this sample of five ASIA A thoracic SCI patients the anatomical extent of the SCI varied. The length of spinal cord discontinuity was 13–60 mm, with the median found at 30 mm. All patients had their spine previously internally stabilized with titanium instrumentation.

Comparing Neurophysiology and MRI

The number of denervated segments was plotted against the length of the spinal cord discontinuity as judged by MRI. The Pearson product-moment correlation coefficient for no of denervated segments and length of lesion was $r = 0.97$, $p < 0.01$.

PAPER II – SEGMENTAL DIAMETERS OF THE HUMAN SPINAL CORD

Published measurements can be combined by adjusting craniocaudal position

To estimate the effect of the adjustment of craniocaudal position of measurements, we set up two second-degree polynomial regression models. The R-squared value for Model 1 (uncorrected positioning) was 68.8% and 86.4% for Model 2 (corrected positioning), which was applied to the corrected data. The 95% confidence intervals for the R-squared values were non-overlapping, indicating a robust difference.

All measurements combined to continuous estimates along the spinal cord

To construct continuous population estimates and achieve further smoothing, a generalized additive model was fit to the weighted averages and weighted standard deviations.

The smoothed continuous population estimates of human spinal cord transverse and anteroposterior diameters of the spinal cord showed the expected shape with a marked cervical intumescence and a smaller lumbar intumescence. The anteroposterior diameter decreased throughout the spinal cord. See paper II for figures and tables of the human spinal cord size.

PAPER III – DESIGN OF A GUIDING DEVICE FOR CLINICAL TRIAL

Simulated segmental spinal cord sizes

Two hundred matched spinal cord transverse and anteroposterior diameters was simulated for each segmental level of the human spinal cord based on data from paper II. Varying the correlation of the bivariate distribution for each segmental level (from higher correlation in the lumbar spine to lower correlation in the cervical spine) allowed the simulated data to satisfy not only the targeted distribution of transverse and anteroposterior diameters but also the shape constraint observed in raw data from measurements of real patients (i.e. regardless of variations in size, the cross-section of the spinal cord can never be too "round" nor too "flat"). From the results obtained, the variation between segments of the spinal cord's elliptical shape and size was much smaller in the thoracic cord compared to the cervical and lumbar portions of the spinal cord.

Error between the simulated thoracic spinal cord and guiding devices

The chosen set of seven guiding device sizes was compared to the simulated thoracic segments T2–T12. For each simulated thoracic segment, we calculated the Euclidian distance to the guiding device sizes to find the device that best fit a given simulated thoracic spinal cord segment.The guiding device size termed "normal 1" was the most frequent "best fit," reflecting its centered position over the distribution of thoracic spinal cord sizes and the design choice to focus on having enough head-room to handle intraoperative swelling of the spinal cord due to manipulation.

The mean error-of-fit comparing simulated spinal cord segments T2–T12 to the best elliptical shape was 0.41 mm and 0.36 mm, the median was 0.31 mm and 0.31 mm, and the 95th percentile was found at 1.3 mm and 0.98 mm for transverse and anteroposterior diameter, respectively. The mean, median, and 95th percentile of the Euclidian distance was 0.60, 0.48, and 1.63 respectively. The mean and median area mismatch was 14.23 mm^2 and 10.93 mm^2 respectively, and the 95th percentile was found at 41.40 mm^2.

The vector model of the human thoracic spinal cord

The variations in white-to-grey matter delineation in segments T2 to T12 were small both within subjects and between subjects. The angle of the dorsal horn increased in the cranial direction in both

subjects, and the transverse distance between the anterior grey matter on each side was slightly different between subjects.

The spinal cord interfaces and graft channels of the guiding device

Using the vector model of the human thoracic spinal cord, we designed a set of channels connecting the 2 spinal cord interfaces with each other for placement of the autologous peripheral nerve grafts. A device design was reached for capturing the majority of corticospinal axons at the cranial interface of the device and guiding them obliquely to grey matter at the caudal interface.

The alignment of guiding device channels and spinal cord white matter tracts

By comparing the white-to-grey matter delineation from the vector model scaled to the sizes of the simulated spinal cord segments and overlaid with the best-fitting device size, we made a qualitative assessment of the alignment between the guiding device channels and the spinal cord white matter tracts.

PAPER IV – ADVERSE EVENTS FROM AN ONGOING CLINICAL TRIAL

Neurological Outcome During the First Two Months

In the first six subjects operated upon, glial scar resection and implantation could be performed without deterioration of neurological function as determined by neurological examination. No significant autonomic changes were seen except a transient increase in sweating in some subjects. Perioperative morbidity had a profile that could be expected considering that the trial includes a surgical intervention in a vulnerable patient group. The adverse events (AEs) outside the expected possible complications to surgery were postoperative aseptic meningitis after one week in two subjects and markedly reduced spasticity in one subject, both AEs are detailed further below. The short-term safety and tolerability merits continuation of the study, and the results obtained indicate that provided the use of precise preoperative and intraoperative monitoring, glial scar resection and implantation can be performed in a selected patient group without deterioration of neurology.

Eligibility and Inclusion

During recruitment to Part A, 22 potential subjects were screened for eligibility. Three subjects were excluded during pre-screening due to unstable mental health (one subject), complex and asymmetrical spinal cord injury (one subject), and lack of consent (one subject). The remaining 19 subjects went through the screening process at Karolinska University Hospital. Ten subjects were excluded during the screening phase due to injury longer than one thoracic spinal cord segment (five subjects), complex and asymmetric spinal cord injury (two subjects), and withdrawal of consent (three subjects).

The remaining nine subjects were randomized to receive either surgery and rehabilitation (six subjects) or rehabilitation only (three subjects). Follow-up and rehabilitation are ongoing and have not been finalized for Part A.

Neurophysiology and imaging during screening

None of the subjects screened in Part A had any remaining voluntary function below the injury level as determined with EMG, transcranial MEP, and transcranial SEP. None of the subjects who underwent functional MRI demonstrated any signs of residual function.

The intercostal EMG in the injury area distinguished several different patterns. Above the injury, we found normal, voluntary activated motor unit potentials (MUPs). Near the sensory level of the subject, voluntary MUPs disappeared in some subjects above the sensory level, and in some

below. Well below the sensory level, we found MUPs of normal configuration that were not voluntary activated. Instead, these MUPs were activated in concert with spastic activation of the lower limbs of the subject. Near the sensory level, subjects had segments showing signs of denervation with fibrillation potentials and positive sharp waves (indicating a previous axonal nerve injury) as well as signs of re-innervation after peripheral nerve injury with large-amplitude neurogenic MUPs. Some subjects also had completely silent segments in the injury area. Some subjects (5/15) who underwent intercostal EMG were found to have an injury spanning more than one neurological segment and were therefore excluded.

Study Population

Of the nine subjects included in Part A, there were seven males and two females. All subjects randomized to surgery were male. Injury mechanisms in the subjects were motor vehicle accident (n=4), fall (n=3), parachute accident (n=1), and motor-cross accident (n=1). In the surgery group, subjects' ages were 25-56 years (median 30). Surgery was performed at 14-42 months after injury (median 27.5 months). In the control group, injury mechanisms were fall (n=2) and motor vehicle accident (n=1), age was 33-48 years (median 36), and inclusion in the study was 35-57 months after injury (median 46). See table 1 for a summary of the included subjects' demographic characteristics.

Surgery

No AEs were reported during surgery. Intraoperative neurophysiology confirmed findings from the preoperative neurophysiology and was used to guide resection margins of the glial scar. In all subjects, the glial scar was resected to expose what visually appeared as viable spinal cord both above and below the glial scar. The lengths of the devices implanted in the first six subjects were 15, 25, 30, 35, 40, and 40 mm. Median surgical time was 8 h and 51 min (7 h 18 min – 10 h 20 min).

Neurological level was not affected by glial scar resection and implantation

As determined by neurological examination, none of the subjects had a deterioration of neurological function above the complete thoracic spinal cord injury after the surgery with glial scar resection and implantation of device. Sensory levels stayed unchanged during the current reporting period from surgery to complete degradation of the device at 60 days post-surgery.

Serious Adverse Events (SAEs)

No SAEs were encountered during surgery, and no SAEs were encountered during rehabilitation. However, during the first post-operative month, five SAEs were reported in three of the six operated subjects. The SAEs encountered in the operated subjects were as follows: pneumonia, *Clostridium difficile* enterocolitis, two cases of postoperative fever (>39.5°C), headache and elevated white blood cell count in CSF, and one case of hygroma. All five SAEs were considered possibly related to the study procedure, and all five SAEs resolved with no lasting effects on the subjects in whom they presented. One subject in the surgery group had a urological SAE after inclusion but five months before surgery. This SAE was considered unrelated to the study.

One of the subjects randomized to rehabilitation had four SAEs during the follow-up period: rib fracture, pyelonephritis, and two undisclosed SAEs that led to early withdrawal from the study. All four SAEs in the control group were considered not related to the study. See table 2 for a comparison of SAEs between groups.

Length of stay after glial scar resection and implantation of device

Subjects spent 3.5 days on average in the neurointensive care unit (NICU) after surgery for monitoring (range 3-4 days) and were discharged to the rehabilitation clinic after 17 days on average

(range 13-20 days). One subject was readmitted to the NICU during the inpatient period for two days because of fever and headache, and one subject was readmitted to the hospital after discharge due to hygroma.

Glial scar resection and implantation of device resulted in transient, aseptic fever and headache one week postoperatively in some subjects

Two SAEs in two subjects were related to postoperative fever (>39.5°C), headache, and elevated white blood cell count in CSF. Three of the remaining four subjects also reported slight headache, photophobia, and elevation of body temperature for one or two days, starting about one week postoperatively. The two subjects with more pronounced fever and headache underwent lumbar puncture at two occasions each. Testing showed increased white blood cell count in CSF, but no pathogens were found in cultures or using broad-range PCR/ESI-MS (216). The subject suffering from hygroma had underwent two lumbar punctures. Whether the reason for the hygroma was due to the surgery and implantation or due to repeated lumbar punctures is unknown.

Neurogenic pain decreased after glial scar resection and implantation of device

Three out of six subjects had significant neurogenic pain in the zone of partial preservation before surgery. This pain was immediately relieved after the operation, enabling one of the subjects to quit his pain medication with Pregabalin. During the first 60 days, some of the pain returned but was greatly reduced, and no additional pain medication was needed.

Spasticity increased in one subject and decreased markedly in one subject after glial scar resection and implantation of device

One subject reported increased spasticity after surgery and had to start medication with oral Baclofen and Mirabegron to alleviate spasticity and problems with neurogenic bladder. In one subject, spasticity reduced markedly after glial scar resection and implantation of the device and resulted in a flaccid paresis below the level of injury. Oral Baclofen was discontinued.

Ongoing and Possibly Related AEs at 60 days postoperatively

At 60 days postoperatively, there were eight ongoing and possibly related AEs in four subjects. Three subjects experienced increased sweating near or below the neurological level but with continuously decreasing intensity. One subject started an SSRI for depressive symptoms 3 weeks after surgery. At 60 days postoperatively, the symptoms were relieved, but the subject was planned for continued medication for the recommended 6 months. Two AEs were related to significantly decreased spasticity in one subject developing within the first month after surgery with a decrease in reflex erection, and another was related to increased spasticity in another subject. The last ongoing and possibly related AE was reported in a subject where neurogenic pain in the zone of partial preservation (ZPP) had diminished markedly after surgery. After a couple of weeks, the subject experienced a return of some neurogenic pain near the ZPP, albeit at a lower level than before surgery; this was still reported as an AE.

Guiding Device was Completely Resorbed during the First Two Months

CT scans of the device area showed a rapid degradation of the device. At 14 days after surgery, the device showed signs of degradation in all subjects. At 60 days, the device had disappeared in six out of six subjects. In the quantitative image analysis, a peak of high attenuating voxels was seen at around 1500 Hounsfield Units representing the calcium sulphate guiding device material. This peak diminished at 14 days and was completely abolished at 60 days after surgery in all subjects, indicating complete degradation of the guiding device.

PAPER V – A LOW-COST LIGHT-SHEET FLUORESCENCE MICROSCOPE

Only minor modifications to the microarray scanner were needed to create the light-sheet

The laser of the microarray scanner could be started and controlled with minor modifications to the control cable. The galvo-scanner could also be controlled by shorting one IC and inserting a control signal to the logic board of the microarray scanner with a function generator. These two minor modifications enabled the formation of a scanned light-sheet at a fraction of the cost for similar optical parts. Constructing the detection axis of the microscope required more effort because a custom-made imaging chamber was needed to enable immersed imaging. Further, the microscope objective, fluorescence filter, tube lens, and camera had to be added to the scanner. Fortunately, several circular lens holders and a micromanipulator were among the unused parts from the scanner itself after modification and could be used to stabilize and position the detection axis.

Light-sheet dimensions were wider than predicted

The empirical full-width at half maximum of the laser focal spot was 5.3 μm, and the corresponding 1/e2 width was 9 μm. The expected 1/e2 width from an ideal system with the characteristics defined is 5 μm, and the full-width at half maximum is 2.9 μm.

Estimated cost of the microscope was about one tenth of OpenSPIM

We estimate the cost of our microscope to be USD 6,000. For comparison, one recent realization of the OpenSPIM architecture cost about USD 60,000 (27). About half of that cost was for laser and optical components, which could be sourced from the microarray scanner at about USD 1,000. Because most of the parts used in the microscope were already in our lab when we started the construction, our estimate of the cost represents a best-guess based on our searching of sources of used laboratory equipment such as ebay.com. Additionally, depending on location, transportation can be a major part of the cost for the microarray scanner. We bought a used scanner from North America on eBay. The cost of the instrument was USD 200, but total cost including transportation was USD 1,000.

Hypoglossal avulsion injury resulted in a significant proliferation of Iba1+ cells in the hypoglossal nuclei one week following injury

We applied our microscope to a hypoglossal nerve injury model employed in our lab and quantified the increase in immunoreactivity for Iba1 one week after injury. The automatic quantification of images collected using the LSFM showed an approximate doubling of Iba1-positive cells and could be visualized in three dimensions thanks to the optical sectioning and *en bloc* imaging. The image acquisition time for the entire hypoglossal nuclei on both sides in a rat was about 4 minutes for collecting 5000 frames at 2 MP or ~10 billion voxels. The high-speed imaging possible with light-sheet fluorescence microscopy makes quantifications of large tissue volumes possible.

DISCUSSION

The papers in this thesis describe parts of the translational process of a preclinical regeneration strategy for complete chronic thoracic spinal cord injury to a clinical trial (papers I-III), the short-term safety reported in the ongoing clinical trial "Safety and Efficacy of SCo806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects" (paper IV), as well as the construction of a low-cost light-sheet microscope (paper V).

EVALUATING THORACIC MOTOR FUNCTION WITH INTERCOSTAL EMG

In paper I, we report that electromyogram (EMG) of the intercostal muscles could be used in thoracic SCI to establish the cranial and caudal margins of motor neuron function. This method was also used in paper IV, which confirmed the applicability of the method in a larger study group and its usefulness in evaluating patients for eligibility in a clinical trial involving patients with thoracic spinal cord injury. Neither paper I nor paper IV has found any adverse effects of the method other than local pain from the EMG needle. Spastic activation of motor-evoked potentials a couple of segments below the injury has been possible in all patients investigated. In the injury zone, most study subjects have shown signs of denervation, and the extent of the denervation in number of segments is consistently in agreement with the craniocaudal extent of the spinal cord injury on MRI. It should be noted that the method does not discriminate between incomplete and complete injuries since it does not target long fiber tracts crossing the injury zone. Also, sensory and autonomic functions are not evaluated.

We argue that interventional studies targeting thoracic spinal cord injury should use intercostal EMG to evaluate motor function precisely. When extrapolating findings from interventions in the thoracic spinal cord to the cervical spinal cord, it is of utmost importance to identify whether an intervention has a negative effect on *local* motor function. Theoretically, an intervention could improve distal functions such as movement of legs or urogenital function but still show a local negative effect in the injury area with loss of motor function in that specific area. If such an intervention was applied to a cervical injury or high thoracic injury, important hand functions might be lost, negating any positive distal effect seen from the treatment. The risk of such mistakes could be reduced by applying the method described in paper I in clinical trials involving thoracic spinal cord injuries.

COMPARING STUDIES ON HUMAN SPINAL CORD CROSS-SECTIONAL SIZE

In paper II, we gather data on segmental cross-sectional size of the normal human spinal cord from all available published sources and created continuous estimates along the craniocaudal axis. Various methods were used in these different studies, including radiological *in vivo* measurements and post-mortem examinations of spinal cord size. Because different studies used diverse anatomical landmarks for the measurements, we created a conversion table between neuronal spinal cord segments and vertebral bony segments to enable convergence of the data on a common craniocaudal axis. We found that the conversion table aligned the cervical enlargement in a statistically superior way as compared to using raw segmental data to align segments. Data on the cervical spinal cord were more abundant, and the proportion of *in vivo* measurements was greater compared to the thoracic and lumbar spinal cord. In the sacral and lumbar spinal cord, the shape changes rapidly along the cranio-caudal axis. Therefore, studies using vertebral bony segments as a landmark utilize an insufficient sampling frequency to capture the change in size accurately in this part of the spinal cord, and the increase in standard deviations reported by us and others in this part of the cord should probably be interpreted to reflect measurement error rather than actual biological variation. To our

knowledge, paper II is the first study that combines measurements from several published studies on the spinal cord within a common craniocaudal axis. Therefore, the resulting estimations in size and variability of the human spinal cord should be of interest to future research in which the dimensions of the spinal cord are of importance (e.g. interventional or diagnostic studies on spinal cord pathology).

Refining and validating the conversion table between vertebral bony segments and spinal cord neuronal segments hold promise for future use as a radiological aid to predict the actual relationship between clinical neurological findings in a patient and radiological pathology in the spinal cord. A major hurdle to accomplishing this is that without knowledge of the exact demarcation of the injury in the cord, correlating findings with neurological level and validating the method are challenging. To overcome this hurdle, analysis of spinal cord injuries resulting from injuries like stab wounds where the location of the pathology can be exactly defined is a possible solution.

One study on spinal cord size that should have been included was found after publication (217). Although it is unfortunate that the study was not included, the results from this study agree with the data generated from our meta-analysis and would not have changed means and confidence intervals significantly.

DESIGNING A GUIDING DEVICE FOR TRIAL IN COMPLETE THORACIC SCI

In paper III, we describe the design and sizing of the guiding device used in paper IV. The design was based on the concept of white-to-grey matter guiding shown to be effective in preclinical studies (170,173,174,183,184) applied to human spinal cord anatomy. The placement of guiding channels was based on degeneration studies and observations from highly localized injuries to the spinal cord such as stab injuries. Recent advances in high-field magnetic resonance imaging have produced anatomical images of spinal cords *ex vivo* with spatial resolution down to 50 um (218). The same study showed sufficient resolution in diffusion tract imaging and tractography to inform possible future designs. If in the future such high-field imaging is possible in patients despite artifacts produced by metal in spinal instrumentation, guiding devices could be manufactured and chosen before surgery based on the size and anatomy of the specific patient. At present, image quality in MRI of SCI patients cannot determine the exact size of the cord near the injury because of metal artefacts; we therefore chose to define a set of device sizes covering the probable spinal cord sizes encountered based on data from paper II. We used the data from paper II to simulate spinal cord sizes and reported the distributions of relevant measurements with the error-of-fit between our set of devices and the simulated spinal cords.

We also added three larger devices to account for swelling of the spinal cord during surgery. Swelling of the cord was not observed in the first six operations performed in paper IV, and the smaller sizes of the devices have been used more often, possibly reflecting atrophy after spinal cord injury (219).

The simulations presented and the approach to evaluating device sizes could be used to inform further research in which a direct physical interaction with the spinal cord is required such as bridging approaches or injection of cells.

SAFETY ASSESSMENT AND SURGICAL METHOD IN A PIG MODEL

An important step in the translational process not detailed in this thesis was the application of the clinical guiding device (paper III) loaded with FGF1 and nerve grafts in a pig model of thoracic spinal cord resection to establish the surgical method and evaluate short-term safety. The data were collected by an external monitor according to good laboratory practice. The study showed no unexpected adverse effects of the procedure, and results are in preparation for publication (220).

EARLY ADVERSE EVENTS IN THE ONGOING CLINICAL TRIAL

After establishment of preclinical efficacy and dosing (183,184,186), refinement of clinical tools for patient selection and evaluation (paper I), adaption of the guiding device to human dimensions (paper II and III), evaluation of the surgical method and safety in a large animal model (220), and securing of funding and formal approval, the study "Safety and Efficacy of SC0806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects" was started with the pharmaceutical company BioArctic AB as sponsor. This randomized, rehabilitation-controlled phase I/IIa trial is the first clinical trial in humans investigating glial scar resection and implantation of a biodegradable guiding device containing peripheral nerve grafts and fibroblast growth factor 1 (FGF1) in complete (AIS-A) thoracic spinal cord injury.

In paper IV, we report adverse events in the first six subjects operated upon and the three subjects undergoing rehabilitation only serving as controls. Rehabilitation is ongoing, and the primary endpoint for part A has not been reached. We present adverse events during the first 60 days postoperatively, and to increase transparency we also describe all *serious* adverse events encountered to date (November 2018) in the study. Further, the degradation of the guiding device as judged by computed tomography scans are presented.

Glial scar resection and implantation could be performed without deterioration of neurological function, despite the need to place the cranial resection margin into structurally normal spinal cord tissue. Considering that the trial includes a surgical intervention in a vulnerable patient group, the majority of adverse events had a profile that could be expected. The adverse events outside the expected possible complications to surgery were postoperative aseptic meningitis after one week in two subjects and markedly reduced spasticity in one subject. The cases of aseptic meningitis resolved without any lasting effects on the subjects.

Interestingly, three out of six subjects who pre-operatively reported chronic neurogenic pain in the injury zone were all relieved of pain in the immediate (60 days postoperatively) period after resection of the glial scar. This suggests that the neurogenic pain in these subjects had some correlation to the structural changes found in a spinal cord injury. Degradation of the guiding device was observed to be complete at 60 days postoperatively as judged by computed tomography.

Taken together, the short-term safety and tolerability were considered acceptable for continuation of the clinical trial.

CONSTRUCTING A DIY LIGHT-SHEET FLUORESCENCE MICROSCOPE

In paper V, we describe the construction of a low-cost but capable light-sheet fluorescence microscope from an outdated micro-array scanner. We applied the microscope to an experimental CNS-injury model used in our lab and automatically quantified the increase in microglia following the injury. Imaging time for capturing the relevant tissue volume at a resolution suitable for automatic cell counting was just a couple of minutes, demonstrating the feasibility for *in toto* imaging of all replicates in a larger experiment. The main obstacle we encountered for extended use of the light-sheet fluorescence microscope has been the difficulty in acquiring consistent immunostaining and transparency of biological tissue across different antibodies and injury types. Many biological questions can still be answered in a more efficient manner with two-dimensional microscopic techniques because it allows faster processing tissue and easier handling of data from microscope to publication.

To realize the potential of light-sheet microscopy beyond beautiful images, consistent contrast generation and tissue transparency have to be combined and applied to unanswered biological questions inaccessible by traditional thin sectioning such as the complex network architecture of the CNS.

ETHICAL ASPECTS OF CLINICAL TRIALS IN SPINAL CORD INJURY

Thanks to the improvements in care and treatments of medical problems after SCI during the 20th century, SCI has transformed into a non-lethal chronic condition, albeit with significant effects on neurological function and health-related quality of life. Therefore, in the view of the author, SCI is *not* a condition for which investigation of new invasive interventions outside the rigorous framework of clinical trial can be motivated.

Clinical trials have the disadvantage of slow processes and high cost, but the process also has numerous important advantages. Key aspects of clinical trial design have evolved over time in research and health care systems to introduce new treatments in a safe and reliable manner. Informed consent from study subjects is a cornerstone of all clinical research, especially those involving interventions. Ethically sound clinical trials go to great length ensuring adequate participant information, rigorous documentation of the consent, as well as the possibility for the study subject to opt out at any time in the process. Formal approval by independent ethical committees and review by medical products agencies ensure that unbiased professionals review the underlying evidence for potential benefits and risks for study subjects. The publication of study protocols and primary endpoints before starting the study ensures that statistical significance in outcome measures are valid and not a result of datamining among many potential candidate outcomes. The use of randomization to intervention or control groups ensures that estimation of effect size is not due to differences in baseline characteristics or other effects of inclusion in a research study.

Despite every effort to ensure safety in study subjects, many treatments tested in clinical trial will lack efficacy, and some will even show harm. Therefore, the utilization of structured reporting of adverse events and independent monitors of safety during the study are pivotal for promoting unbiased evaluation of adverse events and the need for early study termination. If performed in a rigorous manner, even failed trials can still add important information to the field and inform further research.

Invasive procedures in the acute phase of SCI pose a significant challenge in regard to patient safety because at present no method for early evaluation of SCI patients can predict the clinical course with certainty. An early intervention thus poses the risk of harm to the patient beyond the initial injury. In the chronic phase, however, the neurological outcome of the patient is known, and any changes from that level of functioning, deterioration, or improvement can probable be attributed to the intervention. The most important ethical dilemma in the chronic stage is the physical risk that an intervention exposes the study subject to, as well as psychological risk in terms of hope of improvement.

COMPLETENESS OF SCI IN THE ERA OF SPINAL CORD STIMULATION

The concept of "complete" SCI according to the AIS definition is based solely on clinical neurological examination by a skilled health care professional observing and interviewing patients with regard to their conscious connection with body parts below the neurological level of injury. This definition of completeness is adequate and highly relevant in the rehabilitation setting because it clearly predicts the impact of the injury on activities of daily life. From an academic standpoint, however, this view of completeness has been challenged for decades, showing that some patients classified as AIS-A still could exert subclinical but undoubtedly voluntary modifications of movement such as spasticity or reflexes below the neurological level of injury (221). It was also recently shown that some AIS-A patients respond to sensory stimulation below the level of injury when investigated with functional MRI (222). As for being subclinical, the concept of "discomplete" spinal cord injury has no practical implications for the patient because residual function is so limited, and neither *did* it have implications for the indication for interventions such as resection of an injured spinal cord segment

("cordectomy"), a procedure shown to be beneficial in a group of patients with progressing post-traumatic syringomyelia (223). However, several recent publications on epidural electrical stimulation of the lumbar spinal cord (224,225) have put new light on the earlier purely academic question of "discomplete" SCI. When stimulating the lumbar spinal cord within a set of defined parameters, facilitation of voluntary function has been seen, as well as the ability for activation of spinal pattern generators and stepping patterns (224,225).

In the ongoing clinical trial detailed in paper IV in this thesis, study subjects have undergone functional MRI and have also been investigated with motor-evoked potentials and sensory-evoked potentials during screening. These additional investigations of the study subjects minimize the risk for inclusion of a patient with subclinical residual function and a potential future benefit from epidural stimulation. Additionally, all meaningful recovery of function through epidural stimulation has so far been limited to patients with incomplete injuries as classified with neurological examination. Nevertheless, the results from epidural stimulation in chronic SCI clearly highlight the potential benefits of a regeneration strategy able to restore *some* axonal connection between the disconnected caudal spinal cord and the part of the central nervous system still under voluntary control.

THE POTENTIAL OF OUTDATED SCIENTIFIC EQUIPMENT

In a time of increasing consciousness of the negative environmental impact of human activity on our planet, the advantage of reusing products at the highest possible level of functioning has clear benefits. Even though the micro-array scanner used in paper V has played its role as a scientific instrument in its original design, the optical and electrical components were fully functional, and specifications were comparable with identical parts that can be purchased new at a much higher price and environmental impact. If put through the garbage-collecting system (which is often the case when old scientific equipment is no longer useful in the lab), the complex and ingenious micro-array scanner transforms into 100 kg of metal waste polluted with rare trace elements from electronic components, plastics, and glass. This is an unnecessary destruction of resources if components can be reused.

A major hurdle to the efficient reuse of old scientific equipment is the unwillingness of manufacturers of equipment to publish service manuals and codes for communication and programing of microcontrollers in the instruments through the serial interface that is usually present. With this knowledge, the light-sheet microscope in paper V could have been constructed at a fraction of the time because reverse engineering of basic functions would not have been necessary, and most of the microscope could have been controlled by software without modification of the hardware. It is obvious that private companies need to protect their intellectual property in development and during commercialization of products, but in the late phase of the life-cycle of products, releasing detailed information about the instruments would significantly facilitate the recycling of scientific equipment.

FUTURE PERSPECTIVES

The secondary injury ensuing after the initial trauma in SCI has been a target for intervention for a long time because of the feasibility-in-treatment window and the clearly positive effect found in countless of preclinical experimental studies. The ever-expanding knowledge of the complex biology underlying the fine-tuned balance between rescuing uninjured function and sacrificing regeneration results in new potential targets for future treatments at a high pace. When considering the diverse panorama of injuries encountered in the clinical setting and the multifaceted response to injury in the CNS, the failure to show efficacy in clinical trials in SCI over the last decades is unsurprising. The positive effect shown in a controlled preclinical injury leading to a clinical trial probably helped a subset of patients in the trials but had no effect or even showed harm in patients with a different injury biology. The combined result was failure to show efficacy in the primary endpoint of the trial.

Several promising strategies including non-pharmaceutical protocols are reaching phase III at present (159). In combination with better diagnostic tools such as biomarkers and more advanced MRI, these steps indicate that *some* intervention will show benefit in *some* subset of patients in the near future. This would be an important milestone in the decades of preclinical and clinical work that so far have not resulted in any undisputed high-level evidence for *any* intervention in acute SCI except support of vital parameters and prevention of medical complications. Finding an intervention for acute SCI probably would not mean total return of pre-injury function in all patients, but it would result in a gradual shift of demographics from complete and severe injuries to an even higher number of incomplete injuries than what is seen today in the clinic (42).

Although important to find treatments for secondary injury, primary prevention can often be less expensive and seldom receives the focus it deserves. In the elderly with weaker bone due to osteoporosis, it has been shown that the impact of a backward fall on the buttocks can be enough to sustain a burst fracture in the spine; simply lowering the stiffness of the floor or providing other low-tech protection is enough to make this injury type unlikely (36).

Although a further shift in demographics toward incomplete injuries is likely in the future in high-income regions, the long life expectancy after SCI means the shift will be slow; additionally, low income regions that are currently experiencing an increase in motor-vehicle transportation will most likely experience an increase in spinal cord injuries in the coming decades (38,52). Unfortunately, the health care systems in these regions initially lack the resources to supply newly introduced and patent-protected interventions, delaying the benefit of hard-earned results for dissemination to all potential patients and in turn keeping demographics in these regions similar to what we have seen in, for example, Sweden during the last decades.

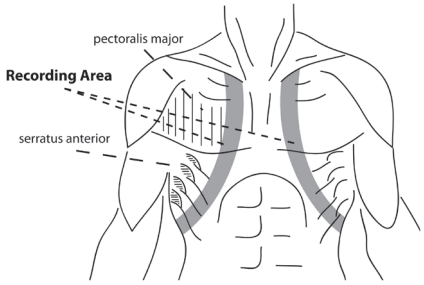
Advances in acute management and interventions in SCI will not improve neurological function for patients living with chronic SCI. The only hope for improvement of neurological function in the chronic state can be found in facilitation of residual function, as has been shown with epidural stimulation, or through regeneration, as has been shown in numerous preclinical projects and a number of case reports in uncontrolled clinical trials.

Possibly, the return of *meaningful* neurological function in severe injuries will require a combination of a regeneration strategy and facilitation of function provided by the relatively small number of regenerate fibers by epidural spinal cord stimulation.

Finally, new targets for improving axonal regeneration after trauma constantly emerge from the expanding preclinical knowledge of CNS injury biology (226). At present, it is unknown whether the regeneration strategy in the ongoing clinical trial described in this thesis will lead to relevant functional recovery after chronic SCI in patients. The methods and results described herein should however be of relevance in the case of other future attempts at spinal cord regeneration after SCI.

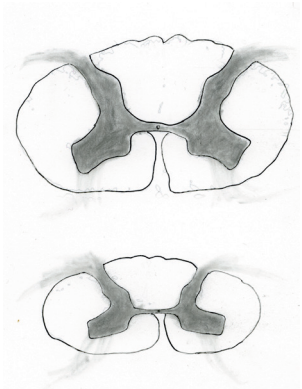
CONCLUSIONS

I



Intercostal EMG could precisely define motor level in thoracic SCI

II

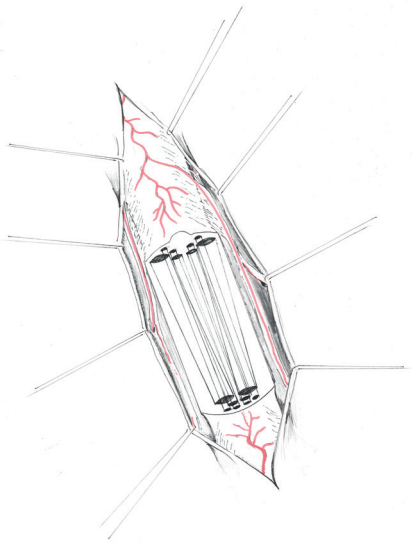


Knowledge on human spinal cord size could be combined using a novel conversion table

III

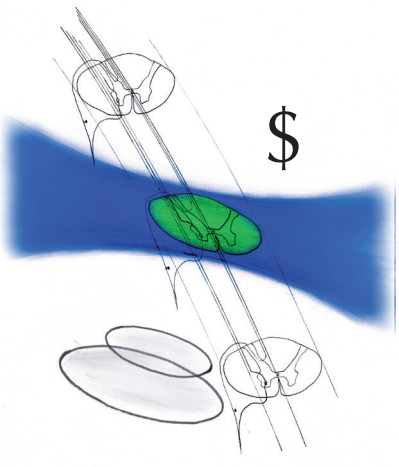
A guiding device for clinical trial was designed

IV



Short-term safety and tolerability of glial scar resection and implantation was acceptable for continuation of the clinical trial

V



An affordable light-sheet microscope was built from a micro-array scanner

CONCLUSIONS

The papers in this thesis describe parts of the translational process of a preclinical regeneration strategy for complete chronic thoracic spinal cord injury to a clinical trial (papers I-III), the short-term safety reported in the ongoing clinical trial "Safety and Efficacy of SCo806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects" (paper IV), as well as the construction of a low-cost light-sheet microscope (paper V).

In conclusion, reaching clinical trial in a translational process is a significant and collaborative undertaking requiring co-operation of multiple institutions, multiple professions, multiple funding sources and rigorous external control of data quality to ensure safety of study subjects. The papers in this thesis details some relevant steps necessary for clinical translation of regeneration strategies in chronic SCI.

Specifically, we conclude that:

1) Both cranial and caudal demarcation of a thoracic spinal cord injury can be defined with electromyography of intercostal muscles (paper I).

2) Using a conversion table between spinal cord neuronal segments and vertebral segments, data on human spinal cord size from different published sources could be combined in a meta-analysis (paper II).

3) A set of spinal cord injury guiding devices of seven sizes can cover the variability of human thoracic spinal cord segments T2–T12 with an acceptable error-of-fit for the elliptical shape as well as guiding channels (paper III).

4) From the first six complete (AIS-A) thoracic spinal cord injury subjects operated on in the ongoing clinical trial "Safety and Efficacy of SCo806 (Fibroblast Growth Factor 1 and a Device) in Traumatic Spinal Cord Injury Subjects," we conclude that with precise preoperative and intraoperative neurophysiology, surgery and implantation can be performed without negative effects on neurological level and safety and tolerability are acceptable to merit the continuation of the study (paper IV).

5) A cost-effective light-sheet microscope could be constructed by modification of an outdated microarray-scanner. At this point, limitations on usefulness were primarily found in methods for tissue preparation and data analysis (paper V).

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